Cellular mechanisms involved in bone cell function and survival

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CELLULAR MECHANISMS INVOLVED IN BONE CELL FUNCTION AND SURVIVAL

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ABSTRACT

Bone remodelling is a dynamic process influenced by factors which effect bone cell activity and survival. This thesis details experiments that investigate some of the cellular mechanisms influencing the formation and activity of osteoclasts and also osteoblast survival and activity.

The role of growth factors, cytokines and osteotropic hormones on osteoblast survival were investigated *in vitro*. Osteoblast survival was promoted by insulin-like growth factor I (IGF-I), IGF-II, insulin and basic fibroblast growth factor (bFGF). Platelet-derived growth factor (PDGF) had no effect on osteoblast survival on its own but potentiated the survival-promoting effects of insulin and IGFs -I and -II. A similar effect occurred when bFGF was added in combination with either IGFs or insulin. The effects of the IGF-I and -II were blocked by an antibody to the type I IGF receptor which also blocked the potentiating effects of PDGF on IGF-I mediated osteoblast survival. Tumour necrosis factor-α (TNF-α) was the only endogenous agent that enhanced programmed cell death (PCD) in this study.

The capability of osteoblasts to support one another's survival in an autocrine fashion was investigated *in vitro*. Osteoblasts survived for 6 days in culture at high cell density in the absence of other cell types, serum or exogenous proteins, but died with the morphological features of apoptosis in these conditions at low cell density. Addition of cysteine to low density cultures enhanced survival during the first 2 days of culture. Catalase also protected osteoblasts in low density cultures whereas superoxide dismutase had no effect. Conditioned medium from high density osteoblast cultures prevented osteoblast apoptosis in low density cultures, as long as antioxidants were present. These results indicate that antioxidants are required for osteoblast survival and that they enhance growth factor mediated survival.

The cellular actions of Interleukin (IL)-11 on bone resorption were investigated using *in vitro* bioassays. IL-11 dose-dependently stimulated release of ^{45}Ca from calvarial explants and increased osteoblast-mediated type I collagen degradation which was prevented by the matrix metalloproteinase (MMP) inhibitor, CT1166. IL-11 had no effect on isolated osteoclast activity even in coculture with primary osteoblasts. In bone marrow cultures, IL-11 dose-dependently increased the number of tatrate-resistant acid phosphatase-positive osteoclast-like multinucleate cells and the surface area of osteoclast

lacunar resorption. The effect of IL-11 on lacunar resorption was prevented by a combination of inhibitors of 5-lipoxygenase and cyclooxygenase. In 17-day-old metatarsal bones, IL-11 prevented the migration of preosteoclasts to future resorption sites, whereas their fusion was unaffected. These results provide evidence that IL-11 stimulates bone resorption by enhancing osteoclast formation and osteoblast-mediated osteoid degradation.

The expression and functional analysis of members of the ADAM (A disintegrin and metalloproteinase) family in bone cells were studied by reverse transcriptase-polymerase chain reaction (RT-PCR), *in situ* hybridization and by antisense technology using bioassays for osteoclast formation and osteoblast differentiation. RT-PCR analysis demonstrated expression of ADAMs -9, -12 and -19 in osteoblasts. *In situ* hybridization studies using DIG-labelled riboprobes revealed expression only of ADAM-12 in isolated osteoclasts and in osteoclasts present in 17-day-old fetal mouse metatarsals. Disruption of ADAM-12 expression in osteoblasts, using antisense constructs had no effect on osteoblast differentiation, assessed by bone nodule formation *in vitro*. In isolated osteoclast cultures, on ivory slices, disruption of ADAM-12 by addition of oligodeoxynucleotides had only minor effects on pit formation whereas in bone marrow cultures osteoclast formation was inhibited. Addition of a recombinant ADAM-12 cysteine rich domain to M-CSF selected bone marrow cells cultured in the presence of M-CSF and RANKL also inhibited osteoclast formation. These results provide strong evidence that ADAM-12 may play a key role in osteoclastogenesis possibly by mediating fusion of osteoclast precursors.

The role and sites of action of serine proteinases (SPs) in bone resorption were investigated in bioassays for osteoclast formation, activity and migration. The SP inhibitors, aprotinin and α_2-antiplasmin dose-dependently inhibited ^{45}Ca release from neonatal calvarial explants. Neither inhibitor influenced either osteoclast formation or resorptive activity. Both inhibitors dose-dependently inhibited the degradation of both type I collagen fibres and non-mineralized bone matrix by murine osteoblasts. In 17-day-old fetal mouse metatarsal explants aprotinin produced a 55% reduction in the migration of osteoclasts from the periosteum to the mineralized matrix after 3 days in culture, however after 6 days in culture the inhibitors were without effect on osteoclast migration. *In situ* hybridization studies demonstrated that tPA and uPA are expressed in mature osteoclasts disaggregated from 6-day-old murine long bones and uPA is expressed in osteoclasts present in 17-day-old fetal metatarsal explants.

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LIST OF ABBREVIATIONS

A disintegrin and metalloproteinase..... ADAM

bovine serum albumin..... BSA

colony stimulating factor..... CSF

Connaught Medical Research Laboratories..... CMRL

deoxy-uridine 5'-triphosphate ..... dUTP

Ethylene-diamine-tetraacetic acid..... EDTA

extracellular matrix..... ECM

fetal calf serum.....FCS

fibroblast growth factor.....FGF

glyceraldehyde-3-phosphate dehydrogenase..... GAPDH

interleukin-11.....IL-11

insulin-like growth factor.....IGF

minimum essential medium.....MEM

matrix metalloproteinase.....MMP

3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide..... MTT

osteoprotegerin.....OPG

osteoprotegerin ligand.....OPGL

parathyroid hormone..... PTH

plasminogen activator..... PA

polymerase chain reaction..... PCR

platelet derived growth factor..... PDGF

tissue inhibitor of metalloproteinase..... TIMP

tumour necrosis factor alpha..... TNF-α

terminal deoxynucleotidyl transferase..... TdT

TdT-mediated dUTP-biotin nick end labelling..... TUNEL

receptor activator of NF-kappaB ligand..... RANKL

secreted placental alkaline phosphatase..... SPAP

transforming growth factor-beta..... TGF-β

T-cell receptor activator induced cytokine..... TRANCE

Trichloracetic acid..... TCA

1,25-dihydroxy-vitamin D..... $1,25\text{-}(OH)_2D_3$

1. GENERAL INTRODUCTION

Bone is a highly specialized tissue resulting mainly from the activities of osteoblasts and osteoclasts. The functions of bone are (*i*) mechanical support of soft tissues, (*ii*) release of calcium for the maintenance of a constant ionic environment in the extracellular fluid, and (*iii*) housing and support of haemopoiesis.

Morphologically two types of bone can be recognized at the macroscopic level: (1) trabecular or cancellous bone, also called spongy bone, comprising 20% of the adult skeleton and (2) compact or cortical bone comprising 80%. Trabecular bone consists of a network of interconnecting bone spicules, the spaces between the network being filled with marrow. Trabecular bone is present predominantly in the metaphysis of long bones. In this way rigidity of structure is attained with minimum weight. Compact bone consists of a dense mass to ensure maximal ability to withstand compression and is found, for example, in the diaphysis of long bones.

Microscopically two types of bone can be recognized: woven bone and lamellar or fine bone. Woven bone is laid down initially during intramembraneous ossification and during fracture repair and is subsequently replaced by lamellar bone during bone remodelling. Compact bone consists of circumferential layers of lamellar bone. The outer circumferential lamellae are covered by a layer of dense connective tissue called the periosteum while the endosteum covers the inner aspect of bone in juxtaposition to the bone marrow cavity. Between the inner and outer lamellar bone are haversian systems each comprising of a central canal containing blood vessels. The cellular component of bone comprises osteoblasts (bone forming cells), osteocytes, bone lining cells and osteoclasts (bone resorbing cells).

1.1 BONE CELLS

Skeletal development and subsequent turnover of bone (remodelling) throughout adult life is established by the tightly regulated interactions between osteoblasts and osteoclasts.

1.1.1 Osteoblasts

Osteoblasts are derived from commited cells of the mesenchymal lineage called osteoprogenitor cells (figure 1-1). Friedenstein (1987) demonstrated that the colony-forming unit-fibroblast (CFU-F), arising from cultures of bone marrow, when implanted into rats in diffusion chambers gave rise to multiple differentiated lineages including osteoblasts as well as chondrocytes, adipocytes and fibroblasts. Thus the CFU-F contains populations of cells that are restricted to differentiation down specific pathways including the osteogenic pathway. Beresford *et al.* (1994) showed the formation of differentiated lineages *in vitro* from bone marrow stromal cultures. The existence of osteoprogenitors in bone marrow stromal cell and calvarial cell cultures that are capable of differentiating into mature osteoblasts has been demonstrated by the formation of bone nodules in these cultures. Using nodule formation as a bioassay it has been estimated that osteoprogenitors are present at a frequency of less than 1% in rat calvarial populations (Bellows and Aubin, 1989).

Four distinct stages in osteoblast differentiation have been identified based on morphological and histochemical criteria: preosteoblasts, mature osteoblasts, osteocytes and bone lining cells (Aubin, 1998). Preosteoblasts are present in the adjacent layer of cells from the osteoblasts present at the bone forming surface and are considered as the precursor of the mature osteoblast. Morphologically active mature osteoblasts are cuboidal cells found in contact with the osteoid (Holtrop, 1977). Intracellularly, osteoblasts have a well developed, extensive rough endoplasmic reticulum and mitochondria indicative of the high metabolic activity of the cell. Mature osteoblasts are characterized by their synthesis and secretion of membrane-bound alkaline phosphatase, type I collagen and other organic components of the bone matrix such as osteocalcin, osteonectin, osteopontin and bone sialoprotein (BSP). In addition osteoblasts express growth factors such as Transforming growth factor-β (TGF-β), Insulin-like growth factors (IGFs) and possess receptors for parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) and 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$).

Current research has focussed on transcriptional control of osteoblast differentiation. Core binding factor 1 (Cbfa1) has been identified as an osteoblast specific transcription factor that acts as a "master" switch for osteoblast differentiation (Ducy *et al.*, 1997). The importance of Cbfa1 in osteogenesis was shown in Cbfa1-deficient mice in which osteoblast

differentiation never occurs (Komori *et al.*, 1997). These Cbfa1-deficient mice have a normally patterned skeleton with an absence of ossification.

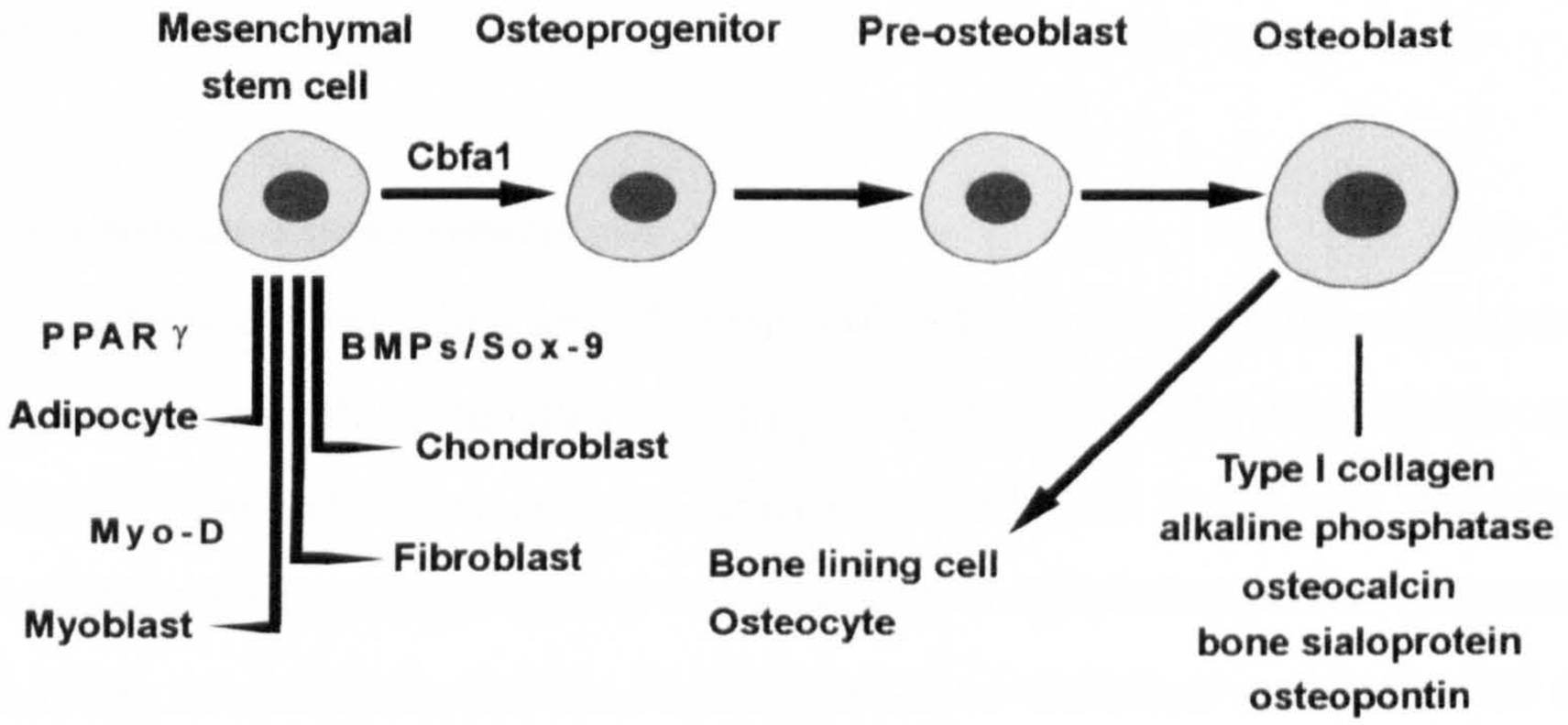


Fig. 1-1 Scheme of osteoblast differentiation.

In this pathway osteoblasts are derived from pluripotent mesenchymal stem cells under the control of the differentiation factor Cbfa1. Mesenchymal stem cells are capable of differentiation into the lineages shown. Peroxisome proliferator-activated receptor γ (PPAR-γ) and Myo-D are transcription factors that control commitment to adipocytes and myoblasts respectively. Commitment to the chondroblast lineage is controlled by bone morphogenetic proteins (BMPs) and the transcription factor Sox-9.

1.1.2 Osteocytes

Osteocytes represent the terminal and most mature differentiation stage in the osteoblastic lineage and arise when osteoblasts incorporate themselves into the newly formed bone matrix. Osteocytes are found embedded in small osteocytic lacunae and possess many cellular processes forming a network of small canaliculi. This extensive network extends throughout the bone matrix forming a communication network between osteocytes and osteoblasts. Osteocytes have reduced levels of alkaline phosphatase and reduced synthesis of macromolecules compared with osteoblasts. Functionally, it is thought that osteocytes may act as sensors of mechanical stress in bone (Kawata and Mikuni-Takagaki, 1998) although the precise role for osteocytes has yet to be elucidated.

1.1.3 Bone lining cells

Bone lining cells are flat elongated cells found lining bone surfaces during phases of quiescent bone remodelling and are considered to be inactive osteoblasts. Dobnig and Turner (1995) proposed that these lining cells can be reactivated by appropriated stimuli such as PTH.

1.1.4 Osteoclasts and bone resorption

Osteoclasts are the principle cells responsible for bone resorption and have several unique ultrastructural characteristics (Holtrop and King, 1977). Osteoclasts are large multinucleated giant cells that contain between 2 and 100 nuclei per cell; they have abundant mitochondria, a large number of vacuoles and lysosomes and an extensive Golgi apparatus. They are rare cells and are found attached to endosteal and periosteal surfaces. The most characteristic feature of osteoclasts is the presence of a deeply infolded finger-like plasma membrane adjacent to the bone surface known as the ruffled border. In actively resorbing osteoclasts the area underneath the ruffled border is known as the sub-osteoclastic resorption zone (SORZ), that becomes acidified by transport of protons across the ruffled border which is mediated by a vacuolar type proton ATPase pump (Väänänen *et al.*, 1990). The ruffled border is surrounded by a clear zone that is organelle-free, consisting of numerous cytoplasmic actin filaments, known as the actin ring, that has been observed in actively resorbing osteoclasts (Väänänen and Horton, 1995) and mediates attachment of the osteoclast to the bone surface. Osteoclasts express abundant $\alpha_v\beta_3$ vitronectin receptors (Nesbitt *et al.*, 1993) present in the clear zone that recognize proteins containing the RGD peptide sequence and are essential for inducing osteoclast polarization. Biochemical analysis has shown that osteoclasts also express the $\alpha_2\beta_1$, collagen integrin receptor, and $\alpha_v\beta_1$ which is another vitronectin receptor (Nesbitt *et al.*, 1993).

Osteoclasts express a number of cell surface antigens in common with cells of the macrophage/monocyte lineage. Osteoclasts strongly express CD45 (also known as the common leukocyte antigen, Athanasou *et al.*, 1987) and a number of other antigens including CD13, CD15, CD68 and CD54. Osteoclasts do not express CD11a, CD11b, CD14 or CD18 (Athanasou and Quinn, 1990).

Osteoclasts are rich in certain enzymes and enzyme histochemistry is commonly used to identify osteoclasts. Osteoclasts are rich in the acid phosphatase isoenzyme, tartrate

resistant acid phosphatase (TRAP; Minkin, 1982) which is a convenient cytochemical marker for osteoclasts although it is not osteoclast specific. Carbonic anhydrase II is expressed at high levels in resorbing osteoclasts and provides the source of hydrogen ions for acidification of the SORZ (Väänänen, 1984). Proteolytic enzymes expressed by osteoclasts that are more specific markers include cathepsin K (Drake *et al.*, 1996) and MMP-9 (Tezuka *et al.*, 1994b).

Osteoclasts have been shown to express high levels of the tyrosine kinase proto-oncogene pp60c-src (Horne *et al.*, 1992) which is expressed only at very low levels in other bone cells. Preferential expression of pp60c-src has been demonstrated on the ruffled border (Tanaka *et al.*, 1992) and transgenic mice lacking the pp60c-src gene developed severe osteopetrosis (Soriano *et al.*, 1991) due to impaired formation of a ruffled border.

Osteoclasts are derived from cells of the monocyte-macrophage lineage (figure 1-2) and investigations have shown that transcription factors play a critical role in osteoclast differentiation. The proto-oncogene c-*fos* is a member of the activator protein-1 (AP-1) transcription factor complex (Angel and Karin, 1991) which includes the Fos-related proteins (Fra-1, Fra-2 and FosB) and Jun-related proteins (c-Jun, JunB and JunD). Wang *et al.* (1992) demonstrated that c-*fos*-deficient mice developed severe osteopetrosis caused by a defect in osteoclast progenitors. Macrophage differentiation was normal and transplantation of normal bone marrow cells into *c-fos*-defective mice rescued the osteopetrosis (Grigoriadis *et al.*, 1994). Osteoclast differentiation was blocked at the point between monocyte-macrophages and osteoclasts. Fleischmann *et al.* (2000) have shown that Fra-1 can rescue this block. Recently it has been shown that the myeloid and B-cell specific transcription factor PU.1 is critical for osteoclast differentiation (Tondravi *et al.*, 1997). PU.1 deficient mice were osteopetrotic with an absence of both osteoclasts and macrophages. These results suggested that PU.1 regulates the initial stages of macrophage differentiation.

The two most widely accepted means of specifically identifying osteoclasts are the demonstration of calcitonin receptors and the ability to form resorption pits or Howship's lacunae on a mineralized substrate such as bone or dentin (Chambers *et al.*, 1984). The demonstration of receptors that bind calcitonin is considered to be a reliable and highly specific marker of mammalian osteoclasts (Hattersley and Chambers, 1989a).

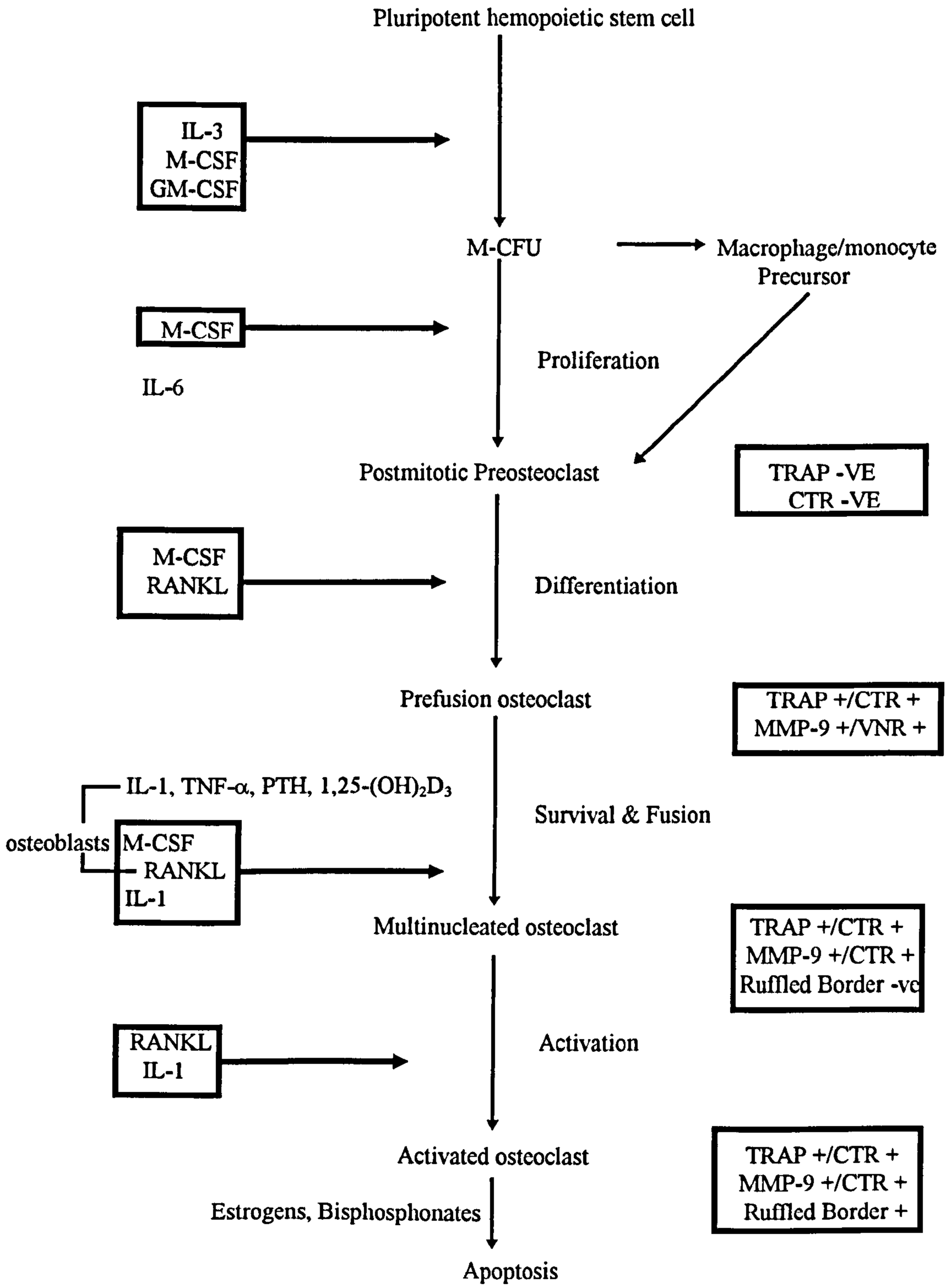


Fig. 1-2 Model of osteoclast differentiation.
The hemopoietic stem cell can give rise to M-CFU and GM-CFU both of which have been shown to give rise to osteoclasts *in vitro* under appropriate conditions. In an alternative pathway GM-CFU/M-CFU can give rise to monocytes and macrophage precursors that can differentiate into osteoclasts.

1.2 ULTRASTRUCTURE OF BONE

Bone matrix consists of both organic and inorganic components. Type I collagen is the most abundant component of the organic phase constituting approximately 90% of the total bone protein (Marks and Popoff, 1988). The remaining 10% of the organic matrix comprises proteoglycans, adhesive glycoproteins and non-collagenous glycoproteins including sialoproteins, osteocalcin and osteonectin. The inorganic component of bone consists of crystalline salts of calcium and phosphate in the form of hydroxyapatite.

1.2.1 Collagens

Type I collagen provides a framework that binds other bone matrix proteins and provides a high degree of tensile strength. Other collagens that are present in trace amounts in bone include types III and V, which together with type I collagen are members of the group of fibrillar collagens consisting of a continuous triple helix assembled into collagen fibrils (Van der Rest and Garrone, 1991). At the molecular level type I collagen has been shown to consist of two $\alpha 1$ chains and one $\alpha 2$ chain each with an N-terminal telopeptide, a long helical domain and a C-terminal telopeptide. The amino acid sequence of the helical domain of fibrillar collagens has been shown to consist of Gly-X-Y repeats, where X is a proline and Y is a hydroxyproline which stabilizes the triple helix (Van der Rest and Garrone, 1991).

1.2.2 Non-collagenous proteins

Adhesive glycoproteins present within bone matrix are characterized by the presence of the amino acid sequence Arg-Gly-Asp (RGD). This confers upon the proteins an ability to bind to the integrin class of cell adhesion molecules (Hynes, 1992). Fibronectin is a 400 kDa adhesive glycoprotein that promotes cell-matrix interactions via the $\alpha_4\beta_1$ integrin in an RGD-independent fashion (Grzesik and Robey, 1994) and has been shown to be upregulated in osteoblasts during bone formation. Vitronectin is a 70 kDa cell attachment protein that is present in bone matrix at low levels where it interacts with the $\alpha_v\beta_3$ integrin (Grzesik and Robey, 1994). Thrombospondin has been shown to mediate adhesion of bone cells and binds to a variety of other matrix proteins such as proteoglycans and fibronectin (Robey *et al.*, 1989).

The sialoproteins are characterized by the presence of sialic acid. Osteopontin is a 32 kDa phosphorylated calcium binding protein present in bone matrix. Expression of osteopontin by osteoblasts is stimulated by 1,25-$(OH)_2D_3$ (Oldberg, 1989) and it is thought that osteopontin may have a role in the binding of osteoclasts to the mineralized matrix of bone via the $\alpha_v\beta_3$ integrin (Miyauchi *et al.*, 1991). Bone sialoprotein-II (BSP-II) is capable of promoting cell attachment via the $\alpha_v\beta_3$ integrin and has a very high affinity for calcium binding; it may play a role in matrix mineralization.

Osteocalcin (bone gla protein, BGP) is a 5.8 kDa protein which accounts for nearly 10% of the non-collagenous proteins of bone extracellular matrix and is expressed by osteoblasts (Owen *et al.*, 1990). The protein belongs to the family of skeletal gla proteins characterized by the presence of three γ-carboxy-glutamic acid (gla) residues formed in a vitamin K dependent posttranslational modification process that enable gla proteins to bind calcium and hydroxyapatite with high affinity. It is thought that the protein functions in the assembly of mineralized bone by regulating hydroxyapatite crystal growth. Further insights into the function of osteocalcin have come from loss of function studies in transgenic mice in which the osteocalcin gene has been deleted (Ducy *et al.*, 1996). These mice show an increase in bone mass as a result of increases in cortical and cancellous bone formation. Osteocalcin may therefore act as an inhibitor of bone formation *in vivo*.

Proteoglycans form part of the remaining 10% of non-collagenous proteins and consist of a core protein to which are covalently attached sulfated glycosaminoglycan (GAG) side chains (Robey, 1996). The proteoglycans decorin (PG-II) and biglycan (PG-I) contain chondroitin sulfate GAGs and have been shown to bind type I collagen and also TGF-β (Takeuchi *et al.*, 1994).

Osteonectin is a 33 kDa protein that is the most abundant glycoprotein present in bone matrix (Tracy *et al.*, 1988). Osteonectin has a high affinity for collagen, calcium and hydroxyapatite and may play a role in mineralization (Bolander *et al.*, 1988).

1.3 BONE REMODELLING

To accomplish its functions, bone undergoes phases of bone resorption followed by bone formation, these two processes constituting bone remodelling (figure 1-3). Bone remodelling is modulated by both systemic and locally produced growth factors (Table 1-1) that effect osteoclast and osteoblast differentiation, activity and programmed cell death.

TABLE 1-1 Hormones, Cytokines and Growth Factors Involved in bone remodelling

Factor	Mr (kDa)	Effect *in vitro*
$1,25\text{-}(OH)_2D_3$[1]		Stimulates bone resorption
PTH[1]	8.5	Stimulates bone resorption
Calcitonin	3.5	Inhibits bone resorption
Estrogen		Inhibits bone resorption
IL-1α[1]	7.5	Stimulates bone resorption
IL-1β[1]	17.3	Stimulates bone resorption
IL-3	14-30	Stimulates osteoclast recruitment
IL-4	15-20	Inhibits osteoclast formation [a]
IL-6	23-38	Induces osteoclast formation
IL-8	6-8	Stimulates osteoclast motility[b]
IL-10	19	Inhibits osteoclast formation[c]
IL-11[1]	11	Induces osteoclast formation
IL-12	70-75	Inhibits osteoclast formation [d]
IL-17[1]	17	Induces osteoclast formation[e]
IL-18	18	Inhibits osteoclast formation
IFN-γ	20-25	Inhibits osteoclast formation [f]
TNF-α[1]	17	Induces osteoclast formation
LIF	58	Induces osteoclast formation
OPG	40	Inhibits osteoclast formation
OPGL[2]	40-45	Induces osteoclast formation
TGF-α	5-7	Stimulates bone resorption
TGF-β[3]	25	Stimulates bone formation and inhibits bone resorption
acidic/basic FGF	16/17	Enhances osteoblast proliferation
IGF-I	7.5	Enhances osteoblast proliferation and
IGF-II	7.5	stimulates bone resorption
PDGF	30	Enhances osteoblast proliferation
M-CSF (CSF-1)	40-90	Enhances osteoclast survival
G-CSF	18-22	No effect in bone marrow cultures
GM-CSF	14-35	Inhibits osteoclast formation

[1]Acts via osteoblasts by inducing expression of OPGL
[2]Also known as T-cell receptor activator induced cytokine (TRANCE), receptor activator of NF-kappaB ligand (RANKL), osteoclast differentiation factor (ODF)
[3] Inhibits osteoclastogenesis by inducing OPG expression and inhibiting OPGL in osteoblasts.
[a]Riancho *et al.* (1993); [b]Fuller *et al.* (1995a); [c]Owens *et al.* (1996); [d]Horwood *et al.* (2001); [e]Kotake *et al.* (1999); [f]Takahashi et al. (1986).

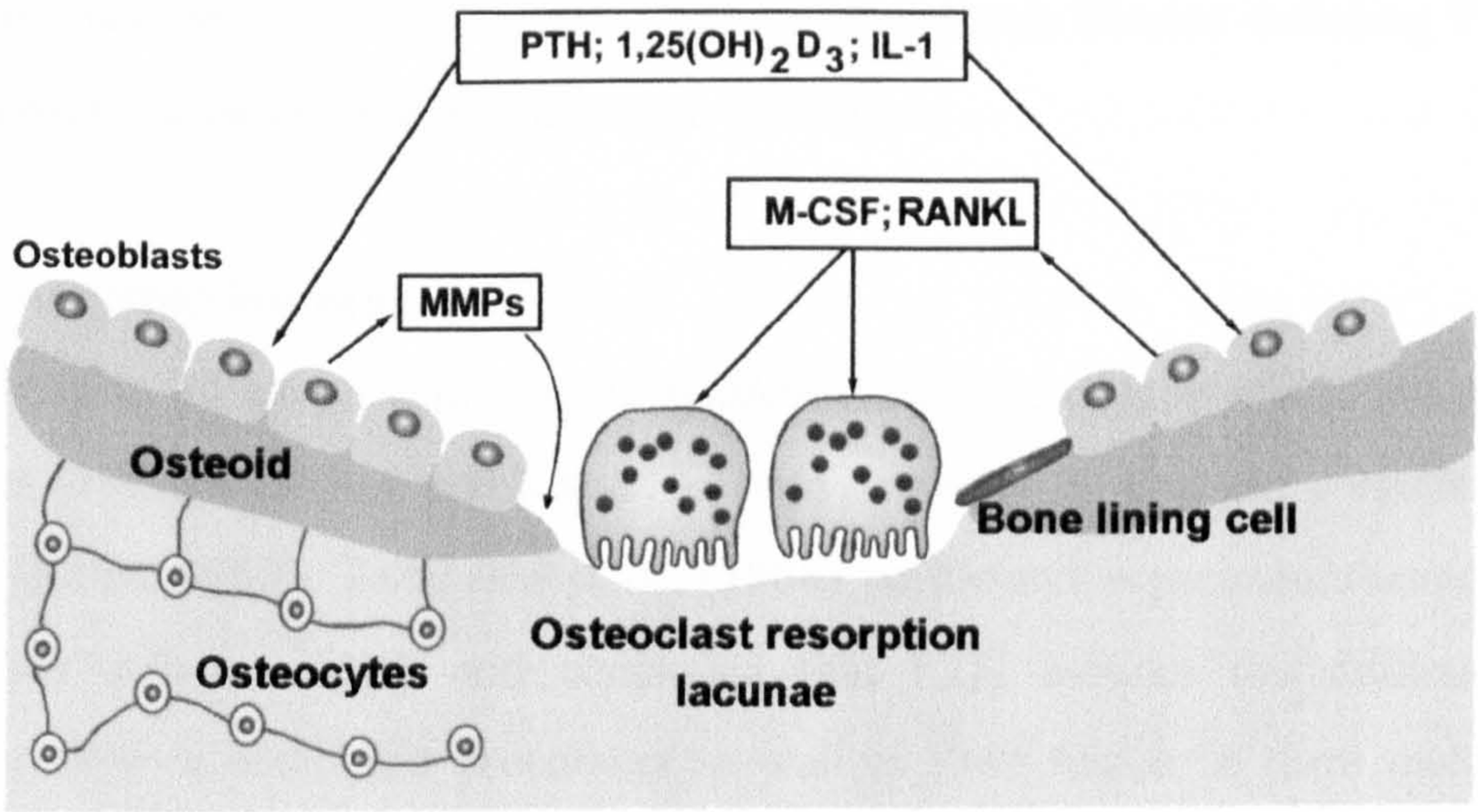


Fig. 1-3. Cellular components of bone.

Interactions between osteoblasts and osteoclasts are tightly regulated by osteotropic hormones such as PTH and 1,25-$(OH)_2D_3$ that stimulate expression receptor activator of nuclear factor (NF)-κB ligand (RANKL) by osteoblasts as well as matrix metalloproteinases (MMPs) that degrade unmineralized osteoid.

The resorptive phase involves a complex cascade whereby osteoclasts are recruited and subsequently migrate to future sites of bone resorption. Following dissolution of the mineralized matrix, osteoclasts may undergo programmed cell death (PCD). Growth factors released from the mineralized matrix during bone resorption stimulate bone formation by osteoblasts, which subsequently become entrapped in the matrix to form osteocytes or may die by PCD. Bone remodelling takes place initially in embryogenesis in the formation of the long bones during endochondral ossification. In this process osteoclasts present in the periosteum of the primitive long bone migrate through a thin layer of osteoid into the calcified cartilage that is partially resorbed to form the future bone marrow cavity. Osteoblasts are then recruited and form a collar of woven bone by a process of intramembraneous ossification that becomes the future midshaft of the long bone. This layer of woven bone is further resorbed later in development and replaced with lamellar bone. Bone remodelling continues throughout adult life, the two processes of bone formation and resorption being coupled by growth factors.

1.3.1 Systemic Factors regulating bone remodelling

Bone remodelling is regulated by numerous systemic factors including PTH, 1,25-$(OH)_2D_3$, insulin, calcitonin and reproductive hormones.

1.3.1.1 Parathyroid Hormone

PTH is an 84 amino acid peptide synthesized by the parathyroid gland in response to decreases in serum calcium levels and has been shown to be a potent stimulator of bone resorption (Raisz, 1965). Takahashi *et al.* (1988) performed experiments using a murine bone marrow culture system and concluded that PTH induces the differentiation of immature to mature osteoclast precursors as well as their fusion to form multinucleated cells (MNCs). McSheehy and Chambers (1986) showed that isolated osteoclast cultures do not respond directly to PTH but do respond to conditioned medium from PTH-treated osteoblasts. Hence, a humoral factor produced by osteoblasts mediates the resorptive effects of PTH on mature osteoclasts. Rouleau *et al.* (1988) confirmed the osteoblast as the target cell for PTH by showing that ^{125}I-PTH binds preferentially to rat osteoblasts and not osteoclasts. More recently, Yasuda *et al.* (1998) have shown that PTH upregulates the expression in osteoblasts of RANKL, that can both activate mature osteoclasts and mediate osteoclastogenesis. Although PTH receptors have not conclusively been demonstrated on osteoclasts, recent studies by Datta *et al.* (1996) have shown that PTH stimulates osteoclasts directly to produce the superoxide radical (O_2^-).

1.3.1.2 1,25-$(OH)_2D_3$

1,25-$(OH)_2D_3$ is the most active metabolite of vitamin D and has been shown to stimulate bone resorption in organ culture (Raisz *et al.*, 1972). Subsequent studies showed that 1,25-$(OH)_2D_3$ stimulates osteoclastogenesis in a murine bone marrow culture system (Takahashi *et al.*, 1988a) and in cocultures of spleen cells and osteoblasts (Takahashi *et al.*, 1988b). In these studies 1,25-$(OH)_2D_3$ induced the formation of TRAP +ve MNCs which satisfied the major criteria for osteoclasts such as the possession of calcitonin receptors. In subsequent studies, Suda *et al.* (1992) showed that cell-to-cell contact was required for 1,25-$(OH)_2D_3$-induced osteoclast formation in the spleen/osteoblast coculture system and suggested that an unknown factor expressed on the plasma membranes of osteoblasts was responsible for mediating 1,25-$(OH)_2D_3$-induced osteoclastogenesis. Yasuda *et al.* (1998) have shown that RANKL exists in a membrane bound form and that its expression is

upregulated by 1,25-$(OH)_2D_3$. Although receptors for 1,25-$(OH)_2D_3$ have not been demonstrated on osteoclasts *in vitro*, Mee *et al.* (1996) used a technique called *in situ* reverse transcription polymerase chain reaction in bone sections to demonstrate the presence of 1,25-$(OH)_2D_3$ receptor transcripts in actively resorbing osteoclasts. 1,25-$(OH)_2D_3$ acts on cells of the osteoblast lineage to produce several non-collagenous proteins, including osteocalcin (Price and Baukol, 1980) and matrix Gla protein (MGP; Fraser *et al.*, 1988).

1.3.1.3 Calcitonin

Calcitonin is a thyroid peptide hormone consisting of 32 amino acids, the major action of which appears to be a direct inhibition of osteoclast-mediated bone resorption (Reynolds and Dingle, 1968). High-affinity receptors for calcitonin are found in large numbers on the cell membrane of osteoclast precursors and mature osteoclasts (Lee *et al.*, 1995). Binding of calcitonin to its receptor on osteoclasts results in increased adenylate cyclase activity and cAMP accumulation. Calcitonin has been shown to cause detatchment of osteoclasts from bone surfaces (Nicholson *et al.*, 1986) and it can inhibit recruitment of osteoclasts from bone marrow cultures (Takahashi, 1988a). To examine how calcitonin inhibits osteoclast function, Suzuki *et al.* (1996) investigated calcitonin-induced morphological changes of the cytoskeleton of osteoclasts. Calcitonin disrupted actin rings and inhibited pit formation by osteoclasts placed on dentin slices. These changes in the cytoskeleton were mediated by the protein kinase A signal transduction pathway since forskolin, which activates protein kinase A, mimiced the effects of calcitonin.

1.3.1.4 Insulin

Insulin is a key systemic regulator of bone formation (Canalis, 1993c). Lack of insulin, as exemplified by type I diabetes mellitus, is associated with osteoporosis, delayed bone maturation, decreased bone mass, and an overall 2-fold increase in fracture rate (Bouillon, 1989). It is known that mature osteoblastic cells are responsive to insulin. *In vitro* studies using primary isolated osteoblasts or clonal osteoblast cell lines have shown that insulin induces changes in alkaline phosphatase activity (Levy *et al.*, 1986), collagen synthesis (Kream *et al.*, 1985) and cell replication (Hock *et al.*, 1988). Pun *et al.* (1989) showed that the rat clonal osteosarcoma cell line, UMR106 expresses approximately 80 000 high affinity insulin receptors at the cell surface. Studies have shown heterogeneity of

insulin receptor expression within neonatal rat calvaria *in vivo*, and insulin receptor expression and responsiveness is associated with the mature osteoblastic phenotype (Thomas *et al.*, 1996)

1.3.1.5 Reproductive hormones

Bone cells are major targets of estrogen and to a lesser extent progesterone. These hormones play a major role in bone remodelling in the adult skeleton and estrogen deficiency following menopause is associated with accelerated loss of bone and development of osteoporosis. The importance of estrogen to the maintenance of normal bone mineral density has been demonstrated by ovariectomy in rats (Kalu *et al.*, 1991a). In this model ovariectomy results in decreased bone density and reduced cancellous bone volume which can be suppressed by administration of 17-β-estradiol (Kalu *et al.*, 1991b). *In vitro* studies have demonstrated that bone marrow from ovariectomized mice form significantly more TRAP+ve MNCs when cultured with 1,25-$(OH)_2D_3$ than cells from sham-operated mice (Kalu, 1990). Cytokine production by cells in the local bone microenvironment is regulated by estrogen. Pacifici *et al.* (1987) reported increased production of IL-1 by monocytes from patients with osteoporosis and Ralston *et al.* (1990) demonstrated inhibition of TNF-α production by peripheral blood mononuclear cells from postmenopasal women following 17 β-estradiol treatment. Subsequent studies showed that treatment of mouse calvarial cells with estrogen inhibits production of IL-6 (Girasole *et al.*, 1992). Recently, Shevde *et al.* (2000), demonstrated that estrogen suppresses RANKL-induced osteoclast differentiation in murine bone marrow cells which is mediated in part by a repression in the level of c-Jun.

Growth factor expression by osteoblasts has also been shown to be regulated by estrogen. Oursler *et al.* (1991b) demostrated increased expression of TGF-β by primary adult human osteoblasts following estrogen treatment and similarly Slater *et al.* (1994) demonstrated increased expression of TGF-β and IGF-I in cultures of primary human fetal osteoblasts. Recent studies have demonstrated enhanced osteoblastic differentiation, as indicated by bone nodule formation, in mouse bone marrow cultures following treatment with 17 β-estradiol (Qu *et al.*, 1999).

1.3.2 Local factors regulating bone remodelling

During bone formation osteoblasts and other cells within the bone microenvironment synthesize a variety of growth factors and cytokines that are sequestrated by the bone matrix (Canalis, 1993c). A number of polypeptide growth factors are found in abundance in the extracellular matrix of bone and when released during phases of bone resorption these growth factors have an effect on bone cell survival and regulate bone cell function.

1.3.2.1 Prostaglandins (PGs)

PGs are cyclopentanoic acids that are biosynthetically derived from the C_{20} polyunsaturated fatty acid, arachidonic acid, via the cyclooxygenase pathway. Arachidonic acid is present in cell membrane phospholipids and is released primarily through the hormonally regulated actions of phospholipase A_2 (PLA_2; Smith, 1992). It is then converted into PGG_2 by the enzyme prostaglandin G/H synthase (PGHS) of which there are two isoforms, PGHS-1 (Cyclooxygenase-1;COX-1) and PGHS-2 (Cyclooxygenase-2;COX-2). PGHS-1 is constitutively expressed in many tissues (Langenbach *et al.*, 1995) and has recently been shown to be induced in the bone marrow stromal cell line, ST2, by 1,25-$(OH)_2D_3$ and dexamethasone (Adams *et al.*, 1999). PGHS-2 is expressed at extremely low levels in most tissues and is induced by cytokines (Du Bois *et al.*, 1994).

The effects of PGs on osteoclasts are varied. Early studies demonstrated that prostaglandins were potent activators of bone resorption in organ culture (Dietrich *et al.*, 1975). However, when added to isolated osteoclasts *in vitro* it was found that PGs transiently inhibited bone resorption (Fuller and Chambers, 1989b).

Evidence using murine bone marrow cultures indicates that prostaglandins promote the differentiation of osteoclasts from haematopoietic precursors (Akatsu *et al.*, 1989a; Collins and Chambers, 1991). The effects of PGs in these assays were mimicked by dibutyryl-cAMP suggesting a possible role for cAMP in osteoclast differentiation. Furthermore Collins and Chambers (1992) showed that osteoclast formation was stimulated by PGE_2 in cocultures of spleen cells and stromal cell lines. More recent research has shown that PGE_2 stimulates expression of messenger RNA for RANKL in osteoblasts (Yasuda *et al.*, 1998). Furthermore Wani *et al.* (1999) have shown that PGE_2 acts directly on precursors synergistically with RANKL in the induction of osteoclast differentiation and

maturation but does not synergize with RANKL for resorption-stimulation in mature osteoclasts.

1.3.2.2 Receptor Activator of Nuclear Factor (NF)-κB Ligand

RANKL, also called osteoprotegerin ligand (OPGL) and TNF-related activation-induced cytokine (TRANCE), was recently identified independently by two groups (Lacey *et al.*, 1998; Yasuda *et al.*, 1998a) and is an early gene expressed upon stimulation of the T-cell receptor (Wong *et al.*, 1997) and a stimulatory factor for dendritic cells (Anderson *et al.*, 1997). It is expressed by osteoblasts as a membrane-associated factor and also as a soluble factor in response to several osteotropic factors such as 1,25-$(OH)_2D_3$, PTH, and interleukin-11 (IL-11). RANKL deficient mice exhibit severe osteopetrosis and completely lack osteoclasts as a result of an inability of osteoblasts to support osteoclastogenesis (Kong *et al.*, 1999).

In cultures of murine bone marrow and spleen cells RANKL in the presence of macrophage colony-stimulating factor (M-CSF) has been shown to be necessary and sufficient for differentiation of osteoclast precursors into mature osteoclasts (Yasuda *et al.*, 1998a; Lacey *et al.*, 1998; Quinn *et al.*, 1998). In addition, RANKL stimulates ^{45}Ca release by fetal murine osteoclasts in long bone cultures (Tsukii *et al.*, 1998) and stimulates resorption by mature, fully differentiated osteoclasts when cultured on bovine cortical bone slices (Burgess *et al.*, 1999). Fuller *et al.* (1998) have shown that RANKL rapidly increased osteoclast motility and spreading and inhibited osteoclast apoptosis. The receptor for RANKL, receptor activator of nuclear factor (NF)-κB (RANK) is restricted in bone to cells of the osteoclast lineage (Hsu *et al.*, 1999). Signal transduction from RANK may involve members of the TNF receptor-associated factor (TRAF) family since TRAF 1, TRAF 2, TRAF 3, TRAF 5 and TRAF 6 can interact with the carboxyl terminus of RANK (Galibert *et al.*, 1998). However, TRAF 6 deficient mice possess an osteopetrotic phenotype which is different to RANKL and similar to that of c-src (Lomaga *et al.*, 1999).

1.3.2.3 Osteoprotegerin/Osteoclastogenesis Inhibitory Factor (OPG/OCIF)

Osteoprotegerin was isolated as a decoy receptor for RANKL by two groups (Simonet *et al.*, 1997; Yasuda *et al.*, 1998b). High levels of OPG/OCIF are expressed in bone and the steady state mRNA levels are higher than those of RANKL. OPG mRNA has

been detected in osteoblastic cell lines (Hofbauer *et al.*, 1998) and in marrow stromal cell lines (Yasuda *et al.*, 1998b). OPG acts as a soluble decoy receptor for RANKL preventing it from binding to RANK. Thus, the effects of OPG on osteoclastogenesis oppose those of RANKL. OPG-deficient mice exhibit severe osteoporosis as a result of enhanced osteoclast formation and function (Mizuno *et al.*, 1998). It has been shown in bone marrow/stromal cell and spleen/stromal cell cocultures that OPG completely inhibits osteoclast differentiation at concentrations of 10 to 100 ng/ml (Simonet *et al.*, 1997). In addition to inhibiting osteoclast formation OPG has been shown to inhibit osteoclast activity assessed by pit formation but at a higher concentration than is required to inhibit osteoclast differentiation (Tsukii *et al.*, 1998).

1.3.2.4 Interleukin-1

Interleukin-1 (IL-1) is a cytokine produced by monocyte-macrophage cells and marrow stromal cells and exists as two functionally related proteins, IL-1α and IL-1β with molecular weights of 17.5 and 17.3 kDa respectively (March *et al.*, 1985). Both IL-1α and IL-1β are potent osteoclast-activating factors that promote bone resorption both *in vitro* and *in vivo*. Gowen *et al.* (1983) and Heath *et al.* (1985) were the first to show that IL-1 stimulates bone resorption in bone organ culture and that prostaglandin production is partially responsible for mediating the effect of IL-1. Subsequently it was shown that IL-1 stimulates pit forming activity of isolated rat osteoclasts through a soluble factor secreted by osteoblasts. Tsukii *et al.* (1998) subsequently showed that IL-1α stimulated bone resorption was suppressed by OPG implicating RANKL as the mediator for IL-1 stimulated bone resorption. Boyce *et al.* (1989) were the first to show that local injections of IL-1 over the calvaria increase both osteoclastic bone resorption within 24 h and bone formation during the following 3-4 weeks. More recently, IL-1 increased osteoclast formation in human bone marrow cultures which is mediated by PGE_2 (Lader and Flanagan, 1998). Several classes of IL-1 receptors have been identified to date. These include the IL-1 type I receptor (IL-1RI) which has a molecular weight of 80 kDa (Hannum *et al.*, 1990; Eisenberg *et al.*, 1990) and has been shown to be responsible for IL-1 mediated bone resorption (Garrett *et al.*, 1993). IL-1α has been shown to promote the survival of mouse osteoclasts generated *in vitro* (Jimi *et al.*, 1995) and this was due to activation of the transcription factor, NF-κB (Jimi *et al.*, 1998). In addition IL-1 induces multinucleation and

activation of prefusion osteoclasts in the absence of osteoblasts leading to the conclusion that IL-1 is involved in pathological bone resorption (Jimi *et al.*, 1999). It has been shown that TNF receptor- associated factor 6 (TRAF 6) is involved in IL-1 signaling leading to activation of NF-κB (Cao *et al.*, 1996) and TRAF 6 deficient mice exhibit defective IL-1 signaling (Lomaga *et al.*, 1999).

1.3.2.5 Interleukin-6

Interleukin-6 (IL-6) is a glycoprotein produced by many cells in the bone marrow microenvironment including monocyte-macrophages and osteoblasts. Production of IL-6 by osteoblasts has been shown to be stimulated by factors that also enhance bone resorption such as IL-1 and tumour necrosis factor-α (TNF-α; Ishimi *et al.*, 1990). IL-6 initiates its biological response via the IL-6 receptor (IL-6R) complex. IL-6 initially binds to the cell-surface IL-6R (also known as gp80) and the resulting complex then associates with a second, non-ligand-binding, signal-transducing subunit known as gp130 (Yamasaki *et al.*, 1988). This leads to homodimerization of gp130 and the activation of signalling pathways within the cell. IL-6 belongs to a family of cytokines that share gp130 as a common signal transducer; these include interleukin-11 (IL-11), leukaemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1) and ciliary neurotrophic factor (CNTF). IL-6R also exists as a soluble form (sIL-6R) that is produced by proteolytic shedding of the membrane bound form (Croucher *et al.*, 1999). The *in vitro* effects of IL-6 on bone resorption are variable and seem to depend on the assay used and the species. In cultures of newborn murine calvaria IL-6 does not stimulate bone resorption and has inhibitory effects on the resorptive response to PTH and 1,25-$(OH)_2D_3$ (Al-Humidan *et al.*, 1990). However, Ishimi *et al.* (1990) showed that IL-6 had potent resorptive effects in cultures of 17-day-old murine fetal metacarpal and calvaria cultures. Tamura *et al.* (1993) demostrated that IL-6 is a powerful inducer of osteoclast formation in murine bone- marrow cultures in the presence of sIL-6R. In a human system, Flanagan *et al.* (1995) showed that IL-6 could not stimulate bone resorption when non-adherent bone marrow cells were cultured on bone marrow stromal cells and IL-6 could not replace stromal factors required for osteoclast formation. Investigations by Gao *et al.* (1998) demonstrated expression of IL-6R and gp 130 on isolated murine osteoclasts.

In vivo studies have shown that IL-6 deficient mice are protected from bone loss caused by estogen depletion and exhibit increased bone turnover (Poli *et al.*, 1994) whilst overexpression of IL-6 is associated with decreased osteoblast and osteoclast number and suppression of bone turnover (Kitamura *et al.*, 1995). Generation of gp 130 deficient mice exhibit increased osteoclast number and osteopenia (Kawasaki *et al.*, 1997).

1.3.2.6 Interleukin-11

IL-11 is a multifunctional cytokine that was originally identified as the cytokine that could induce the proliferation of IL-6 dependent T1165 hemopoietic cells (Paul *et al.* 1990). Expression of IL-11 is restricted to certain cells of the mesenchymal lineage, such as lung fibroblasts, bone marrow stromal cells, articular chondrocytes and synoviocytes (Maier *et al.*, 1993). Whereas the gene structure and amino acid sequence of human IL-11 are unique from those of IL-6 a number of biological actions of IL-11 are shared with IL-6 Musashi *et al.*, 1991a; Tsuji *et al.*, 1992; Musashi *et al.*, 1991b; Teramura *et al.*, 1992) and both cytokines share gp130 as the common signal transducer (Yin *et al.*, 1993; Neuhaus *et al.*, 1994). Recent studies have suggested that IL-11 is produced by osteoblasts in response to IL-1, TNF, TGF-β, 1,25-$(OH)_2D_3$, and PTH (Girasole *et al.*, 1994). Girasole *et al.* (1994) demonstrated that IL-11 dose-dependently stimulates osteoclast-like MNCs formation in cocultures of mouse osteoblasts and bone marrow cells. Morinaga *et al.* (1998) have demonstrated that IL-11 promoted prostaglandin E_2 (PGE_2) synthesis; thus IL-11 may stimulate bone resorption through a PGE_2 synthesis dependent mechanism.

1.3.2.7 Leukemia Inhibitory Factor

LIF is a single chain glycoprotein that was initially isolated on the basis of its capacity to induce differentiation and suppress proliferation of the murine monocytic leukemia cell line M1 (Tomida *et al.*, 1984). OSM is closely related to LIF and can mimic the activities of LIF *in vitro*. Receptors for LIF are present on osteoblasts (Allan *et al.*, 1990; Bellido *et al.*, 1996) and on osteoclast-like cells derived from human giant-cell tumour (Gouin *et al.*, 1999). In an *in vivo* study Metcalf and Gearing (1989) demonstrated an increase in bone mass with some evidence of cortical bone resorption in mice injected with tumour cells producing high levels of LIF. *In vitro* studies have shown that LIF stimulates bone resorption in neonatal mouse calvariae (Reid *et al.*, 1990). In mouse bone

marrow assays LIF, OSM and CT-1 have been shown to induce TRAP +ve MNCs an effect that is synergistically enhanced by dexamethasone (Richards *et al.*, 2000). In long-term human bone marrow cultures LIF increases the number of MNCs formed that express the macrophage polykaryon phenotype (Heymann *et al.*, 1997).

1.3.2.8 Tumour Necrosis Factor

TNF exists as two forms TNF-α and TNF-β. TNF-α is a cytokine expressed mainly by monocytes and macrophages and is the prototype member of the TNF supergene family of ligands (Lotz *et al.*, 1996). It is a hormone like peptide that enters the bloodstream to alter the biology of distant tissues or it can behave as a paracrine mediator acting locally. TNF-α is a true pleiotropic cytokine with numerous biological effects including cytotoxicity. The biological effects of TNF-α are mediated by two transmembrane receptors termed TNFR1 and TNFR2 (Tartaglia and Goeddel, 1992). TNF-β, previously called lymphotoxin, is expressed by lymphocytes and has similar pleiotropic effects to TNF-α. Both TNF-α and TNF-β have been shown to stimulate bone resorption in organ culture (Bertolini *et al.* 1986). Subsequently, Tashjian *et al.* (1987) showed that TNF induced bone resorption could be blocked by inhibitors of prostaglandin biosynthesis. More recently Lader and Flanagan (1998) demonstrated that TNF-α enhances human osteoclast formation in a human bone marrow system, an effect mediated by PGE_2. Hofbauer *et al.* (1999) have demonstrated that TNF-α increased RANKL mRNA by up to two- three-fold in normal human stromal cells. However, Kobayashi *et al.* (2000) demonstrated that TNF-α could induce osteoclast formation from cultures of macrophage colony-stimulating factor (M-CSF)-dependent precursors by a mechanism independent of RANKL/RANK pathway and dependent on TNFR1-mediated signals.

1.3.2.9 Transforming Growth Factors

The transforming growth factors are a family of polypeptide mediators of cell proliferation and differentiation and exist in two forms: transforming growth factor α and β (TGFα and TGF-β). TGF-β is the prototype member of the TGF-β supergene family and occurs as a homodimeric molecule with a molecular mass of 25 kDa. TGF-β is produced by a number of cell types including bone cells and is the most abundant of the growth factors found in bone (Seyedin *et al.*, 1985). To date five isoforms of TGF-β have been purified

from bovine bone matrix (TGF-β1-5) with no significant differences in their activity (Mohan and Baylink, 1991). TGF-β is secreted by osteoblasts and stored in the bone matrix as a latent high molecular weight complex (100-250 kDa) and upon release from the bone matrix becomes activated by the acidic environment beneath bone resorbing osteoclasts (Oreffo *et al.*, 1989). The effects of TGF-β on bone cell function are wide ranging and complex. It stimulates cell replication in cultures of primary osteoblast like cells (Centrella, 1987) and also stimulates bone resorption in neonatal mouse calvarial cultures by a prostaglandin synthesis-dependent mechanism (Tashjian *et al.*,1985). In contrast to these findings Chenu *et al.* (1988) demonstrated that TGF-β inhibits formation of osteoclast-like cells in long-term human bone marrow cultures. TGF-β1 negatively regulates osteoclastogenesis in murine bone marrow cultures by increasing levels of OPG and decreasing the levels of RANKL by bone marrow stromal cells (Takai *et al.*, 1998). In cultures of hematopoietic cells containing few stromal cells TGF-β enhances osteoclast formation in the presence of M-CSF and RANKL (Sells Galvin *et al.*, 1999; Fuller *et al.*, 2000).

1.3.2.10 Bone Morphogenetic Proteins

BMPs form part of the TGF-β superfamily of related peptide growth factors and are known to have an osteogenic potential (Wozney *et al.*, 1988). The BMP subfamily consists of 15 members and are secreted as 30 kDa homodimers or heterodimers, interconnected by seven disulphide bonds (Wozney, 1989). BMP-2, -4, -5, -6, -7 and -9 have been shown to induce ectopic bone formation when implanted into subcutaneous or muscular sites in rats (Gitelman *et al.*, 1994; Celeste *et al.*, 1994) whilst BMP-13 and -14 have been shown to induce ectopic tendon and ligament in rats (Wolfman *et al.*, 1997).

In vitro studies have demonstrated that osteoprogenitor cells are responsive to the effects of BMPs. Asahina *et al.* (1993) demonstated that cultures of newborn rat calvarial cells showed osteogenic differentiation after treatment with recombinant human BMP-7. Subsequently Rickard *et al.* (1994) demonstrated enhanced levels of alkaline phosphatase and increased levels of mRNA for osteopontin, osteocalcin and BSP in rat bone marrow cultures after stimulation with BMP-2. Fromigue *et al.* (1998) have demonstrated enhanced alkaline phosphatase and osteocalcin levels in cultures of primary human bone marrow stromal cells following treatment with recombinant human BMP-2. The effects of BMP-2

on osteoclast formation have been investigated in mouse bone marrow cultures (Koide *et al.*, 1999). BMP-2 alone had no effect on osteoclast formation though it enhanced osteoclast formation in a dose-dependent fashion in the presence of IL-1α. Abe *et al.* (2000) have demonstrated the requirement for BMP-2 and -4 in osteoclast formation by treating mouse bone marrow cultures with a BMP antagonist called noggin. Addition of noggin to these cultures inhibited osteoclast formation which could be reversed by addition of exogenous BMP-2.

1.3.2.11 Fibroblast Growth Factor

FGF was originally identified as a growth factor for mesenchymal cells (Rifkin and Moscatelli, 1989). This activity is due to two proteins, acidic FGF (aFGF; FGF-1) and basic FGF (bFGF; FGF-2) that share 55% homology in their amino acid sequences and bind to the same receptor (Neufeld and Gospodarowicz, 1986). FGFs also seem to be bone regulating factors since they enhance the proliferation of rat osteoblasts *in vitro* (Canalis *et al.*, 1988). However, continuous FGF treatment reduced the synthesis of alkaline phosphatase (ALP; Rodan *et al.*, 1989) and type I collagen (Canalis *et al.*, 1988) by osteoblasts. Osteoblasts can synthesize two types of FGFs, which persist in the bone matrix (Globus *et al.*, 1989) and are released from bone matrix during phases of bone resorption. Montero *et al.* (2000) have examined transgenic mice deficient in FGF-2. These mice exhibited significant decreases in trabecular bone volume and bone formation rates, providing evidence that FGF-2 helps determine bone mass.

Jimi *et al.* (1996) investigated the effects of bFGF on osteoclast-like cell formation using cocultured mouse bone marrow cells with the mouse stromal cell line, ST2, or with primary osteoblastic cells. In this system bFGF had an inhibitory effect on osteoclast-like cell formation induced by 1,25-$(OH)_2D_3$ and also PGE_2 and IL-11. The effects of bFGF on osteoclast formation seem to be varied depending on the culture conditions. Nakagawa *et al.* (1999a) showed that the inhibitory effect of bFGF on 1,25-$(OH)_2D_3$-induced osteoclast formation was due to suppression of 1,25-$(OH)_2D_3$-induced RANKL production. However, Hurley *et al.* (1998) demonstrated osteoclast formation from murine bone marrow cultures stimulated with bFGF by a prostaglandin dependent mechanism. Subsequently, Nakagawa *et al.* (1999b) showed an increase in the expression of RANKL and COX-2 in bFGF stimulated osteoblasts.

1.3.2.12 Platelet Derived Growth Factor

Platelet derived growth factor (PDGF) is 30 kDa polypeptide first identified as a factor in platelets that allowed the growth of fibroblasts *in vitro*. Further characterization of PDGF demonstrated that it is a potent mitogen for all cells of mesenchymal origin including osteoblasts (Linkhart *et al.*, 1986). PDGF exists as a dimer comprised of two polypeptide chains termed A and B. Independent regulation of the expression of the A and B chains allows for the production of at least three different PDGF-related molecules, the AA and BB homodimers and the AB heterodimer (Hannink and Donoghue, 1989). Studies on the biological activities of PDGF on bone revealed that PDGF stimulates cell replication in bone cells derived from neonatal mouse calvariae (Kasperk *et al.*, 1990). PDGF also stimulates collagen and non collagen protein synthesis in rat calvaria organ cultures (Canalis, 1981). PDGF stimulates bone resorption in mouse calvaria by a mechanism that involves prostaglandin synthesis (Tashjian *et al.*, 1982). Zhang *et al.* (1998) demonstrated PDGF receptors on osteoclasts by immunomicroscopy.

1.3.2.13 Insulin-like Growth Factors

The insulin like growth factors-I (IGF-I) and II (IGF-II) are 7.5 kDa polypeptides, sharing 62% homology, expressed by cells of the osteoblast lineage and found in abundance in bone matrix (Mohan *et al.*, 1993). IGFs are fixed in bone by means of IGF binding proteins (IGFBPs) of which six have currently been characterized (Bautista *et al.* 1991). These proteins modulate the activity of the IGFs at the local level. Although the IGFs are found in abundance in bone matrix the relative concentration of IGF-I is 10 to 15 fold less than that of IGF-II. Similarly IGF-I is produced by human bone cells at 50 to 100-fold less than that of IGF-II (Mohan *et al.* 1988). Both IGF-I and IGF-II have been shown to stimulate cell proliferation in monolayer cultures of human bone cells (Weregedal *et al.*, 1990). In addition Hock *et al.* (1988) showed that IGF-I stimulates matrix synthesis in 21-day-old fetal rat calvaria in organ culture. Mochizuki *et al.* (1992) demonstrated that IGF-I but not IGF-II stimulates bone resorption through its direct or indirect action of supporting the generation and activation of osteoclasts. More recently Hill *et al.* (1995a) demonstrated that bone resorption induced by IGF-I and IGF-II is mediated by osteoblasts.

1.3.3 Colony Stimulating Factors (CSFs)

The colony stimulating factors are macromolecules that influence the proliferation and differentiation of multipotential haemopoietic stem cells into haemopoietic progenitors that are commited to a specific lineage. These progenitors further differentiate under appropriate signals into terminally differentiated myeloid cells (Metcalf, 1985). The CSFs are glycoproteins that have been characterized on the basis of their ability to promote the formation of colonies of multipotential stem cells in semi-solid media. The family of CSFs include interleukin-3 (IL-3), macrophage colony stimulating factor (M-CSF, also named CSF-1), granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF). Since osteoclasts are derived from progenitors of the macrophage/monocyte cell lineage, CSFs are likely to play a role in osteoclast formation as shown in Fig. 1-2.

1.3.3.1 Macrophage colony stimulating factor (M-CSF)

M-CSF exists in a soluble form and a membrane bound form that are encoded by a single gene which undergoes alternative splicing. The biological effects of M-CSF are mediated by a high affinity receptor of the tyrosine kinase family which is encoded by the protooncogene *c-fms* (Fixe and Praloran,1997). Osteoblasts have been shown to produce M-CSF *in vitro* and this is stimulated by PTH, IL-1 and TNF-α (Felix *et al.*, 1989). Using murine metatarsals as an *in vivo* model for osteoclastogenesis, Hofstetter *et al.* (1995) demonstrated that M-CSF production by osteoblasts was associated with the final stages of osteoclast formation.

Evidence implicating M-CSF as an important growth factor in osteoclastogenesis has come from studying animal models which have a defect in the M-CSF gene resulting in an osteopetrotic phenotype. Such models include the murine *op* mutation (Felix *et al.*, 1990b; Wiktor-Jedrzejczak *et al.*, 1990) in which the defect is caused by a point mutation within the coding region of the M-CSF gene resulting in a defective protein (Yoshida *et al.*, 1990). Phenotypically *op/op* mice are characterized by a virtual absence of osteoclasts (Marks and Lane, 1976). Administration of recombinant human M-CSF to *op/op* mice induced osteoclast formation and susequent restoration of bone resorption, thus providing unequivocal proof that the defective M-CSF in the *op/op* mice is the cause of the osteopetrosis (Felix *et al.*, 1990b). Takahashi *et al.* (1991) confirmed that osteoclast

formation was dependent on M-CSF and that the *op* defect was in the stromal cells. In coculture experiments, osteoblasts from phenotypically normal animals were able to support osteoclast formation when cocultured with precursors from *op/op* mice. However, osteoblasts derived from *op/op* mice were not able to support osteoclast formation when cultured with precursors from either *op/op* or normal mice. Investigations into the action of M-CSF on mature osteoclasts cultured on bone slices have demonstrated an inhibitory effect of M-CSF on osteoclast resorptive activity (Hattersley *et al.*, 1988). This was subsequently shown to be due to M-CSF inducing spreading and acting as a chemotactic agent (Fuller *et al.*, 1993) thus reducing the proportion of resorbing osteoclasts. Confirming this Weir *et al.* (1994) demonstrated that addition of anti-M-CSF antibodies to fetal rat long bone cultures led to an increase in PTH-stimulated bone resorption. In cultures of isolated human osteoclasts M-CSF increased bone resorption which was attributed to increased osteoclast survival (Edwards *et al.*, 1998).

1.3.3.2 Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF is a 124 amino acid cytokine that stimulates the proliferation of granulocytes and macrophages and is expressed by various cell types including T-lymphocytes, macrophages and endothelial cells. Treatment of whole calvaria and primary mouse osteoblasts with PTH or TNF-α stimulates the production of GM-CSF (Felix *et al.*, 1988; Horowitz *et al.*, 1989).

The effects of GM-CSF on osteoclast formation are equivocal. MacDonald *et al.* (1986) demonstrated that GM-CSF, when added to human bone marrow cultures, enhanced osteoclast formation in the presence of 1,25-$(OH)_2D_3$. It was subsequently shown that GM-CSF could induce the formation of TRAP-positive MNCs when added to cultures of human haemopoietic stem cells (Kurihara *et al.*, 1989). Furthermore Takahashi *et al.* (1991) demonstrated that colonies formed in the presence of GM-CSF contain osteoclast precursors. At the same time Abe *et al.* (1991) showed that GM-CSF present in conditioned medium from concanavalin A stimulated spleen cells was an important fusion factor for alveolar macrophages. However, other evidence suggests that GM-CSF is not required for formation of osteoclasts. GM-CSF cannot support osteoclast formation when substituted for M-CSF in coculture of normal spleen cells with *op/op* osteoblasts (Hattersley *et al.*, 1991) and injection of GM-CSF into *op/op* mice cannot reverse the osteopetrotic phenotype

(Wiktor Jedrzejczak *et al.*, 1994). Furthermore, a GM-CSF knock-out mouse is not osteopetrotic (Dranoff *et al.*, 1994). Subsequent investigations showed that GM-CSF inhibited osteoclast formation in bone marrow cultures and suppression of GM-CSF production by dexamethasone resulted in a higher number of osteoclasts (Shuto *et al.*, 1994). Further, Udagawa *et al.* (1997) showed that GM-CSF mediates the inhibitory effects of IL-18 on osteoclast formation. Although GM-CSF has been shown to induce bone resorption in fetal long bone organ cultures (Bertolini and Strassman 1991) this may be due to the release of PGE_2 and other cytokines by activated macrophages.

1.3.3.3 Granulocyte colony stimulating factor (GCSF)

G-CSF is a 25 kDa cytokine that stimulates the proliferation and differentiation of neutrophils and is expressed by activated macrophages, endothelial cells, fibroblasts and osteoblasts. G-CSF has been shown to be produced by calvaria, primary osteoblasts and osteoblastic cell lines treated with TNF-α (Felix *et al.*, 1988, 1991).

Granulocyte colony-forming cells (G-CFC) contain multipotential progenitor cells and it has been reported that (G-CFC) when injected into osteopetrotic *ia* rats can give rise to osteoclasts and thus partially reverse the osteopetrotic phenotype (Schneider and Relfson 1988).

1.3.3.4 Interleukin-3 (IL-3)

IL-3 is a 22-36 kDa glycoprotein that induces the proliferation of early stage multipotential haemopoietic stem cells (Sonoda *et al.*, 1988) that are capable of differentiating into granuloctes, macrophages and erythroid cells. Murine calvaria have been shown to produce IL-3 when treated with lipopolysacharide although primary cultures of osteoblasts do not (Felix *et al.*, 1988).

Cells grown in the presence of IL-3 can differentiate into osteoclasts under a microenvironment provided by osteoblasts (Takahashi *et al.*, 1988). Subsequently, Kurihara *et al.* (1989) demonstrated that osteoclast-like cells could be generated from haemopoietic progenitors in the presence of IL-3 and 1,25-$(OH)_2D_3$ in the absence of any other growth factor. At the same time Barton and Mayer (1989) showed that IL-3 could induce osteoclast formation in a murine bone marrow assay in the absence of 1,25-$(OH)_2D_3$; thus it may be that IL-3 can act on later stages in the formation of osteoclasts. However, the

culture systems used in these cases does not preclude the involvement of other cytokines being produced.

Contrary to the previous studies, IL-3 can inhibit osteoclast formation induced by 1,25- $(OH)_2D_3$ in murine bone marrow cultures (Hattersley and Chambers, 1990; Shinar *et al.*, 1990). Susequently Takahashi *et al.* (1991) demonstrated that if IL-3 is added at the start of the bone marrow culture osteoclast formation was inhibited. However when bone marrow cells were cultured in methylcellulose in the presence of IL-3 and the colonies that formed were then cocultured with osteoblasts, osteoclasts were formed.

1.4 PROTEINASES

Following mineral dissolution during the resorptive phase of bone remodelling organic matrix degradation occurs mediated by proteinases. Two major classes of proteinases, the matrix metalloproteinases (MMPs) and the cysteine proteinases (CPs) are directly involved in digestion of the organic matrix beneath the subosteoclastic resorption zone (Hill *et al.*, 1994a, 1994b). Everts *et al.* (1998) have shown that CPs attack the organic matrix initially prior to digestion by MMPs. In addition MMPs also participate in the migration of preosteoclasts to sites of bone resorption (Blavier and Delaisse,1995). Members of a fourth family of proteinases the ADAMs (a disintegrin and metalloproteinase) have recently been implicated in osteoclast formation by mediating fusion of osteoclast precursor cells (Abe *et al.*, 1999).

1.4.1 Plasminogen Activator /Plasmin pathway

The plasminogen activator (PA)/plasmin pathway is a highly regulated proteolytic pathway that results in the generation of the broad spectrum serine proteinase, plasmin, from the zymogen plasminogen. Activation of plasminogen is achieved by the activities of the plasminogen activators (PAs) of which there are two types: tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA). The PA/plasmin pathway is regulated by four members of the serine proteinase inhibitor (serpin) family. Plasminogen activator inhibitor-I (PAI-I) and plasminogen activator inhibitor-II (PAI-II) regulate the activities of uPA and tPA. A third member of the serpin family, protease nexin-I (PN1) regulates thrombin, plasmin and uPA, whilst α_2-antiplasmin regulates plasmin. The pathway has been implicated in a plasminogen-plasmin-latent metalloproteinase activation cascade in

type I collagen degradation (Thomson *et al.*, 1989) and also in the activation of latent growth factors such as TGF-β (Lyons *et al.*, 1990). Thus the PA/plasmin pathway may play an important role in bone remodelling and in the coupling of bone resorption and bone formation.

uPA is secreted as a precursor protein of 55 kDa and is activated by cleavage into a 30 kDa heavy chain and a 24 kDa light chain linked by a disulphide bond, with the active site residing in the 30 kDa fragment (Wun *et al.*, 1982). Recent studies have demonstrated expression of uPA in osteoclasts by RTPCR of RNA extracted from microisolated mouse osteoclasts (Yang *et al.*, 1997). uPA has a domain structure consisting of a Kringle domain, a serine proteinase-like active site and a growth factor domain (GFD). The noncatalytic NH_2-terminal fragment contains the GFD and Kringle domain and is referred to as the amino terminal fragment (ATF). The GFD of the ATF is necessary for the binding of uPA to its specific receptor.

The urokinase-type plasminogen activator receptor (uPAR) is a glycosylated GPI-linked protein (Ferguson and Williams, 1988) and receptors have been found on fibroblasts and the human monocyte-like U937 cells and have been postulated to have a role in migration of cells through matrices. The receptor functions in the activation and degradation of uPA and pro-uPA bound to its receptor can be activated by plasmin (Cubellis *et al.*, 1989). The uPA/uPAR complex cannot be internalized and degraded (Cubellis *et al.*, 1990).

tPA is secreted as a 72-kDa polypeptide and is activated by cleavage into a 39-kDa heavy chain and a 33-kDa light chain linked by a disulphide bond. tPA has been found in human plasma and tissue extracts as well as in normal and malignant cells. The heavy chain has no proteolytic activity but contains two Kringle domains that assist in binding fibrin to plasminogen (Banyai *et al.*, 1983), a finger domain involved in fibrin binding (van Zonneveld et al., 1986) and a GFD with homology to human and murine EGF. Receptors for tPA consist of two populations, a low affinity and a high affinity population (Barnathan *et al.*, 1988; Hajjar 1991).

1.4.1.1 Regulation of the PA system in bone

Plasminogen activator activity is increased in normal and malignant rat osteoblasts and also in murine calvariae by bone resorbing agents such as PTH, PGE_2 and 1,25-$(OH)_2D_3$ (Hamilton *et al.*, 1984, 1985; Thomson *et al.*, 1989). Hamilton *et al.* (1985)

demonstrated that increased PA activity in normal and malignant rat osteoblasts in response to PTH treatment was due to tPA, as identified by fibrin zymography; there was no evidence for the presence of uPA in conditioned medium. Further studies showed that the stimulation of PA activity by PTH and PGE_2 was mediated by cAMP (Allan *et al.*, 1986). Subsequent investigations showed that stimulation of rat osteoblastic UMR106-01 cells with hPTH (1-34) increased the mRNA for both tPA and uPA two fold with a subsequent increase in activity and decreased mRNA for PAI-I (Fukumoto *et al.*, 1992). Allan and Martin (1995) have shown that PGE_2 increased mRNA levels for tPA, uPA and uPAR in primary cultures of rat neonatal osteoblast-like cells while mRNA for PAI-I was modulated in a biphasic manner and PN1 and PAI-2 were not modulated by PGE_2. Thus, in rat osteoblasts PTH may increase PA activity by decreasing production of PAI-I.

$1,25\text{-}(OH)_2D_3$ has been reported to stimulate plasminogen activator activity in normal and malignant rat osteoblasts by a cAMP-independent mechanism and that the increase in activity is due to inhibition of PAI-I mRNA (Fukumoto *et al.*, 1994)

Studies have shown that various growth factors and cytokines expressed by osteoblasts modulate components of the PA/plasmin system. Cheng *et al.* (1990) have shown that incubation of fetal rat osteoblastic calvarial cells with either aFGF, bFGF, EGF or PDGF elevates the PA activity in the conditioned media as assayed by fibrin zymography. Allan *et al.* (1991) have demonstrated that TGF-β treatment of rat osteoblast calvarial cells or rat osteoblastic UMR 106-01 cells dose dependently inhibited PA activity by increasing PAI-I mRNA levels and protein. Similarly LIF inhibits PA activity in rat osteoblasts by increasing PAI-I synthesis (Allan *et al.*, 1990).

1.4.1.2 Role of the PA/Plasmin system in bone remodelling

The activation of growth factors by plasmin provides a crucial role for the PA/plasmin pathway in osteoblast mediated bone formation. Lyons *et al.* (1990) have demonstrated that latent TGF-β produced by osteoblasts can be activated by plasmin and Yee *et al.* (1993) have shown that treatment of cultures of UMR-106 cells with PTH and plasminogen resulted in a 6-fold increase in the concentration of active TGF-β which was plasminogen dependent. In activating TGF-β the PA/plasmin system provides a mechanism for the coupling of bone resorption with bone formation.

The osteoblast PA/plasmin pathway is involved in the activation of procollagenase secreted by osteoblasts in response to bone resorbing agents (Hamilton *et al.*, 1985). Studies by Thompson *et al.* (1989) found that collagenolysis by mouse calvarial osteoblasts grown on Type I collagen films is plasminogen dependent, implicating the PA/plasmin system as being of importance in modulating the degradation of the osteoid layer covering the bone surfaces. Recent studies have shown that the ability of osteoblasts from tPA:uPA deficient knockout mice to degrade nonmineralized matrix is significantly reduced (Daci *et al.*, 1999).

The role of the PA/plasmin system in osteoclast migration and activity has been investigated using 17-day-old fetal mouse metatarsal explants (Leloup *et al.*, 1994) and also by studying the effects of inactivation of the genes for uPA, tPA and PAI-1 (Leloup *et al.*, 1996). Leloup *et al.* (1994) showed that PTH enhanced the activity of tPA in extracts of metatarsals but not of uPA. Later work showed that PTH increased the activity of tPA in calvariae derived from wild type tPA+/+ and uPA+/+ or deficient uPA-/- and PAI-/- mice; no differences were observed for any parameter of bone resorption between tPA+/+, tPA-/-, uPA+/+ and uPA-/- calvariae (Leloup *et al.*, 1996). Cultures of metatarsals from uPA-/- mice released ^{45}Ca at a slower rate at the beginning of culture. Thus uPA may be important in the migration of preosteoclasts from the periosteum into the calcified cartilage. Daci *et al.* (1999) have shown that osteoclast formation and resorption of mineralized matrix is unaffected by a combined deficiency of both tPA and uPA although nonmineralized matrix digestion is effected.

1.4.2 Matrix Metalloproteinases

The matrix metalloproteinases are a family of Zn^{2+} dependent endopeptidases which collectively are capable of degrading the whole spectrum of extracellular matrix components (Murphy and Reynolds, 1993a). Four major subgroups have been recognized (Table 1-2), the collagenases, gelatinases (type IV collagenases), stromelysins and membrane type MMPs. Within each group there is a characteristic domain structure and a highly conserved amino acid sequence between members of each group. Six domains have been identified and are illustrated in Fig. 1-4.

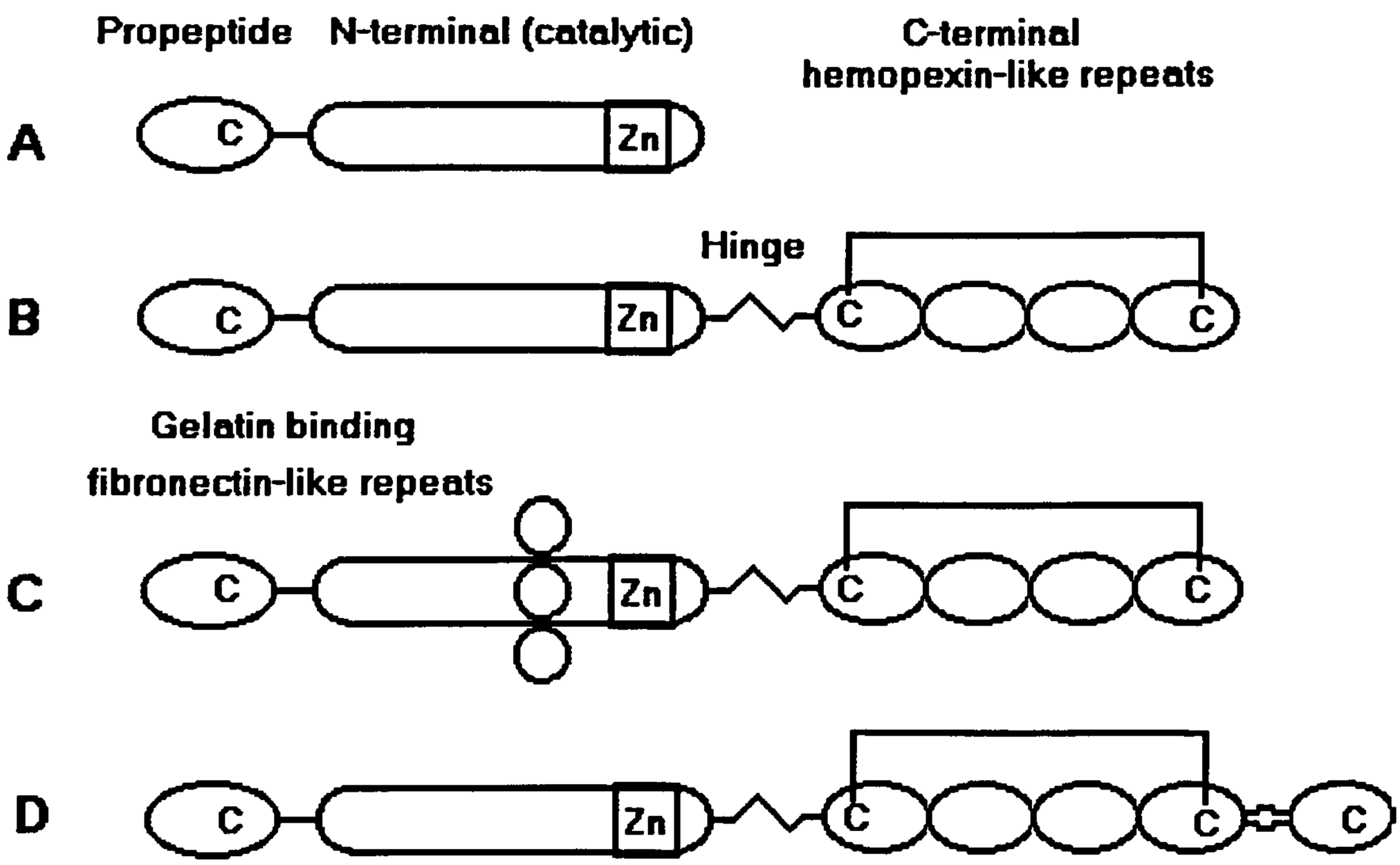
All subgroups of MMPs consist of a propeptide of approximately 80 amino acid residues, and a catalytic domain of about 160 residues. Within the propeptide is the

consensus sequence PRCVNPD forming a cysteine switch mechanism in which the cysteine residue ligands with the active site Zn^{2+} in the catalytic domain, conferring latency on the MMPs (Birkedal-Hansen *et al.*, 1993). Within the catalytic domain is the zinc binding motif HEXGHXXGXXH in which the three histidine residues bind the catalytic zinc.

With the exception of matrilysin, all MMPs possess a *C*-terminal domain which is linked to the catalytic domain by a proline rich hinge region. The *C*-terminal domain consists of hemopexin-like repeats which confers substrate, matrix and inhibitor binding capabilities upon different MMPs. Gelatinase A and B possess a fourth domain consisting of three fibronectin-like repeats confering an ability to bind to gelatin and collagen. The MT- MMPs possess in addition a transmembrane domain with a cytoplasmic tail. Recently Nagasse and Woessner (1999) have identified MMP-23 in which the hemopexin domain has been replaced with a cysteine-rich, proline-rich and interleukin/receptor-like region.

1.4.2.1 Collagenases

Three members of the collagenase subgroup have been identified which have the ability to cleave fibrillar triple helical collagen at neutral pH (Matrisian, 1992). Collagenase-1 (MMP-1, also called interstitial or fibroblast collagenase) is synthesized by a wide variety of cells including fibroblasts, monocytes and macrophages (Welgus *et al.*, 1985) and chondrocytes (Lefebvre *et al.*, 1990). Production of collagenase-1 by osteoblasts has been demonstrated and shown to be elevated in response to bone resorbing agents such as PTH and 1,25-$(OH)_2D_3$ (Heath *et al.*, 1984). Collagenase-2 (MMP-8, also called neutrophil collagenase) is expressed by polymorphonuclear leukocytes (Murphy *et al.*, 1980) and has also been found to be expressed by human chondrocytes (Cole *et al.*, 1996). Collagenase-3 (MMP-13) was reported by Freije *et al.* (1994) to be closely related to the rat and mouse interstitial collagenase (Henriet *et al.*, 1992) and has been found to be expressed by human chondrocytes (Reboul *et al.*, 1996). Immunohistochemical studies have demonstrated collagenase-1 expression in mouse, rat and rabbit osteoclasts (Delaisse *et al.*, 1993; Hill *et al.*, 1994b) and in human osteoclasts *in vivo* (Bord *et al.*, 1996). However, *in situ* hybridization studies could not detect collagenase-1 in osteoclasts and synthetic inhibitors of collagenase had no effect on lacunar resorption by isolated osteoclasts (Fuller and Chambers, 1995b).



A: matrilysin; **B**: neutrophil collagenase, interstitial collagenase, stromelysin -1, -2, -3, metalloelastase; **C**: gelatinase -A and -B; **D**: membrane type metalloproteinase.

Fig. 1-4 Domain structure of matrix metalloproteinases (from Reynolds and Meikle 2000).

Six domains can be recognized. A propeptide containing a conserved cysteine residue at the *C*-terminus which ligands with the active site zinc forming a cysteine switch mechanism and maintaining the protein in a latent configuration. An *N*-terminal catalytic domain which contains the Zn^{2+} binding consensus sequence. A *C*-terminus containing hemopexin-like repeats which are involved in sustrate binding. A proline rich hinge region. A gelatin binding domain comprising fibronectin-like repeats and a transmembrane domain with a ctoplasmic tail.

1.4.2.2 Gelatinases

Gelatinases (type IV collagenases) are involved in proteolysis and disruption of basement membranes by degradation of types IV, V and denatured collagens. There are two types of gelatinases, 72 kDa gelatinase A (MMP-2; Collier *et al.*, 1988) and 92 kDa gelatinase B (MMP-9; Wilhelm *et al.*, 1989).

In bone, gelatinase A is expressed constitutively by cultured human osteoblasts (Rifas *et al.*, 1989, 1994; Meikle *et al.*, 1992, 1995) and rabbit calvarial osteoblasts (Meikle *et al.*, 1994). Immunolocalization studies have demonstrated expression of gelatinase A in rabbit osteoclasts (Hill *et al.*, 1994b) though not in human osteoclasts (Wucherpfennig *et al.*, 1994). Gelatinase B has been shown to be secreted by the human osteosarcoma cell lines (U2OS and MG63; Rifas *et al.*, 1994) but secretion by normal human osteoblasts was not detected. Likewise Meikle *et al.* (1992) found very little immunohistochemical staining for gelatinase B in normal human osteoblasts. Expression of gelatinase B has been noted in mouse osteoclasts (Reponen *et al.*, 1994) and in human osteoclasts by immunohistochemistry and *in situ* hybridization (Wucherpfennig *et al.*, 1994; Okada *et al.*, 1995). Hill *et al.* (1995b) demonstrated, using a synthetic gelatinase inhibitor, that gelatinase A and B are likely to play a role in bone resorption. Further studies by Blavier and Delaisse (1995) showed that gelatinase B is obligatory for the migration of preosteoclasts to the developing bone marrow cavity of primitive long bones.

1.4.2.3 Stromelysins

The stromelysin subgroup of MMPs include stromelysins 1 and 2 (MMP-3 and MMP-10), which are highly homologous and exhibit an ability to cleave a broad spectrum of matrix components including aggrecan, fibronectin, nidogen, laminin and type IV collagen (Murphy *et al.*, 1991; Nagase, 1995). Stromelysin 1 has also been shown to degrade types III, IX and X collagen. Stomelysin 3 (MMP-11) shares less homology to stromelysin 1 and 2 and displays weak catalytic activity against the same substrates (Murphy *et al.*, 1993b).

Mesenchymal cells such as chondrocytes and fibroblasts are commonly found to express stromelysin (Matrisian, 1992). In bone, stromelysin has been shown, by immunohistochemistry, to be produced by human osteoblasts (Meikle *et al.*, 1992) upon stimulation with PTH or monocyte conditioned medium, as well as by mouse osteoblasts

after 1,25-$(OH)_2D_3$ treatment (Thomson *et al.*, 1989). Rifas *et al.* (1994) demonstrated secretion of stromelysin by two human osteosarcoma cell lines (MG63 and U2OS) that was increased upon stimulation with phorbol myristate acetate (PMA), interleukin-1β and tumour necrosis factor α. Expression of stromelysin1 has been noted in osteoclasts (Okada *et al.*, 1995) and stomelysin 2 has been found strongly associated with human osteoclasts in contrast to stromelysin 1 (Bord *et al.*, 1998).

1.4.2.4 Membrane-type matrix metalloproteinases (MT-MMPs)

The membrane-type matrix metalloproteinases are a recently described subset of metalloproteinases characterized by a transmembrane domain anchoring them into the cell membrane. To date five members of the MT-MMP subset have been identified. MT1-MMP (MMP-14) was initially discovered on the surface of invasive tumour cells (Sato *et al.*, 1994; Sato and Seiki 1996) and subsequently found to be expressed in osteoclasts (Sato *et al.*, 1997). Other members are widely expressed in a variety of tissues and include MT2-MMP (MMP-15; Takino *et al.*, 1995), MT3-MMP (MMP-16) and MT4-MMP (MMP-17; Puente *et al.*, 1996) while MT5-MMP (MMP-24; Pei, 1999) is expressed in a brain specific manner. The MT-MMPs exhibit an ability to cleave a broad spectrum of matrix proteins including fibrin, fibronectin, tenascin, nidogen, aggrecan and perlecan, and are also capable of processing proTNF-α to its mature form (d'Ortho *et al.*, 1997).

1.4.2.5 Tissue inhibitors of matrix metalloproteinases (TIMPs)

TIMPs are the major regulators of MMP activity and to date four members of the TIMP family have been identified that possess 12 invariant cysteine residues. TIMP-1 is a 28 kDa glycosylated protein (Docherty *et al.*, 1985) and is produced by many cell types including osteoblasts (Meikle *et al.*, 1992, 1995) and osteoclasts (Hill *et al.*, 1993). TIMP-1 inhibits MMPs by forming a 1:1, high affinity and irreversible non-covalent complex with the active form of MMPs (Willenbrook and Murphy, 1994) and can also form a complex with progelatinase B (Goldberg *et al.*, 1989). TIMP-2 is a 21 kDa unglycosylated protein (Boone *et al.*, 1990) and is constitutively expressed by many cells in culture. TIMP-2 inhibits MMPs in a similar way to TIMP-1 and also forms a complex with progelatinase A. Both TIMP-1 and -2 have been shown to inhibit conversion of progelatinase and prostromelysin to their mature forms. TIMP-2 has been shown to be 2 to 10 times more

effective than TIMP-1 against gelatinase A and B, whereas TIMP-1 inhibits MMP-1 more effectively (Howard *et al.*, 1991). TIMP-3 is a 21-27 kDa protein originally isolated from chicken cells and called ChIMP-3 and cloned from human sources (Apte *et al.*, 1994) and mouse (Leco *et al.*, 1994). TIMP-3 has a wide tissue distribution, with highest expression in the placenta and brain (Apte *et al.*, 1994; Leco *et al.*, 1994) and only low levels found in bone (Leco *et al.*, 1994). Knauper *et al.* (1997) demonstrated that TIMP-3 associates with the C-terminal domain of human collagenase-3. TIMP-3 interacts with gelatinases in a similar manner to TIMP-2 and interacts rapidly with both gelatinase A and B and forms complexes with both progelatinase A and B (Butler *et al.*, 1999). TIMP-4 has recently been identified and cloned from human tissue (Greene *et al.*, 1996) and from adult mouse tissues (Leco *et al.*, 1997) where it has been shown to be more closely related to TIMP-2 and TIMP-3 and expressed in brain, heart, ovary and skeletal muscle. TIMP-4 has been demonstrated to bind to gelatinase A and progelatinase A (Bigg *et al.*, 1997).

1.4.2.6 Activation of MMPs

Most MMPs are secreted from the cell as inactive zymogens (Sellers and Reynolds, 1978) and are activated *in vitro* by proteinases or by exposure to nonproteolytic agents such as organomercurial compounds. *In vivo*, many proMMPs are activated by other MMPs and plasma proteins, thus there exists a number of activation cascades involved in MMP activation (Murphy and Knauper, 1997). Investigations have implicated plasmin, as being of importance in the activation of collaganase, gelatinase B and stromelysin-1 but not gelatinase A in *in vitro* model systems (Murphy *et al.*, 1992). MMP activation by plasmin is initiated extracellularly at or near the cell surface where uPA is bound to uPAR. Sato *et al.* (1994) demonstrated that MT1-MMP is an activator of gelatinase A. More recent studies demonstrating activation of gelatinase A by MT2-MMP (Butler *et al.*, 1997) suggest that other members of the MT-MMP family may be involved in the activation of gelatinase A. This activation cascade requires active MT1-MMP and TIMP-2 bound MT1-MMP (Strongin *et al.*, 1995). MT1-MMP has also been shown to be involved in an activation cascade for human procollagenase-3 which is potentiated by the presence of gelatinase A (Knauper *et al.*,1996) and subsequently involved in an activation cascade for gelatinase-A, -B and collagenase 3 (Cowell *et al.*, 1998).

TABLE 1-2 Subgroups of the matrix metalloproteinase family

Enzyme	MMP No.	Mr (kDa)		Matrix Substrate
		Proform	Active	
Interstitial Collagenase	MMP-1	55	45	Native fibrillar collagens, types
Neutrophil Collagenase	MMP-8	75	58	I, II, III, VII, VIII, X Gelatins
Collagenase-3	MMP-13	60	48	
Gelatinase A	MMP-2	72	66	Type I, IV, V, VII, X, XII, XIV collagen. Gelatin, Elastin, fibronectin.
Gelatinase B	MMP-9	92	86	Type IV, V, VII, X, XIV collagen, gelatin, fibronectin
Stromelysin-1	MMP-3	57	45	Type III, IV, IX, X collagen, aggrecan, laminin, fibronectin
Stromelysin-2	MMP-10	57	44	Type III, IV, V collagen, aggrecan, gelatins, fibronectin
Stromelysin-3	MMP-11	51	44	Procollagens I, II, III
Matrilysin	MMP-7	28	19	Type IV, X collagen, gelatin, aggrecan, proteoglycan laminin, elastin, fibronectin
Macrophage metalloelastase	MMP-12	54	45/22	As for matrilysin
MT1 MMP	MMP-14	66	56	pro-MMP-2,-MMP-13,pro
MT2 MMP	MMP-15	72		MMP-2
MT3 MMP	MMP-16	64	52	MMP-2
MT4 MMP	MMP-17			proTNF
MT5 MMP	MMP-24	63		MMP-2
MT6 MMP (Leukolysin)	MMP-25	34	28	
	MMP-19	56	48	Type IV collagen, laminin, fibronectin, nidogen
Enamalysin	MMP-20			
	MMP-23	44		Unknown
Matrilysin-like	MMP-26	29.6	19	

1.4.2.7 Transcriptional regulation of MMPs

Expression of MMPs in most tissues is low during phases of quiescent ECM remodelling and is induced during periods of active ECM remodelling. MMP gene expression is transcriptionally regulated by a variety of extracellular factors such as growth factors and cytokines (Shapiro, 1998). The promoter regions of MMP -1, -3, -7, -9, -10, -12 and -13 exhibit AP-1 regulatory elements which bind to members of the AP-1 transcription factor family and polyoma enhancer A binding protein-3 (PEA3) elements that interact with members of the Ets transcription factors (Westermark and Kahari, 1999). These regulatory elements play an important role in the regulation of MMP gene expression in response to stimuli including phorbol ester, cytokines and growth factors (Angel *et al.*, 1987). Overexpression of c-fos in transgenic mice has been shown to induce expression of MMP-13 predominantly in bone although expression of MMP-3, -9 and -10 was not affected suggesting that c-fos differently regulates expression of distinct MMP genes *in vivo* (Gack *et al.*, 1994).

1.4.3 ADAM (A Disintegrin and metalloproteinase)

The ADAMs or MDCs (metalloproteinase-like, disintegrin-like, cysteine-rich proteins) form a large family of multifunctional proteins that fall within the metzincin superfamily (Wolfsberg and White, 1996; Primakoff and Myles, 2000). To date 30 transmembrane glycoproteins have been identified (Table 1-3) which have a characteristic multi-domain structure (Fig. 1-5). Tang and Hong, (1999) identified a second group of soluble cell associated ADAMs that have C-terminal thrombospondin repeats and have been termed ADAMTS (Fig. 1-5).

All ADAMs isolated to date consist of approximately 750 amino acids and have a prodomain, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic tail domains. The protease and disintegrin domains of the ADAMs share approximately 30% sequence identity with a group of metalloproteinases isolated from snake venoms known as the reprolysins.

Expression studies of the known ADAM genes have shown that they have a surprising tissue distribution (Table 1-3). A total of 12 ADAM genes are expressed exclusively and 3 ADAM genes predominantly in the testis (Wolfsberg *et al.*, 1995; Hooft

van Huijsduijnen, 1998; Zhu *et al.*, 1999; Cerretti *et al.*, 1999). This suggests a special relationship between ADAM function and the processes of spermatogenesis and fertilization. Cell-cell interactions are believed to play crucial roles in neural function and development and 4 ADAM genes have been found to be expressed in brain tissue. ADAM --22 and -23 (MDC 2 and 3) are expressed exclusively and ADAM-11 (MDC) predominantly in the brain (Sagane *et al.*, 1998). Kuzbanian the Drosophila orthologue of ADAM-10 has been shown to be expressed in neurons and to be involved in neurogenesis (Qi *et al.*, 1999).

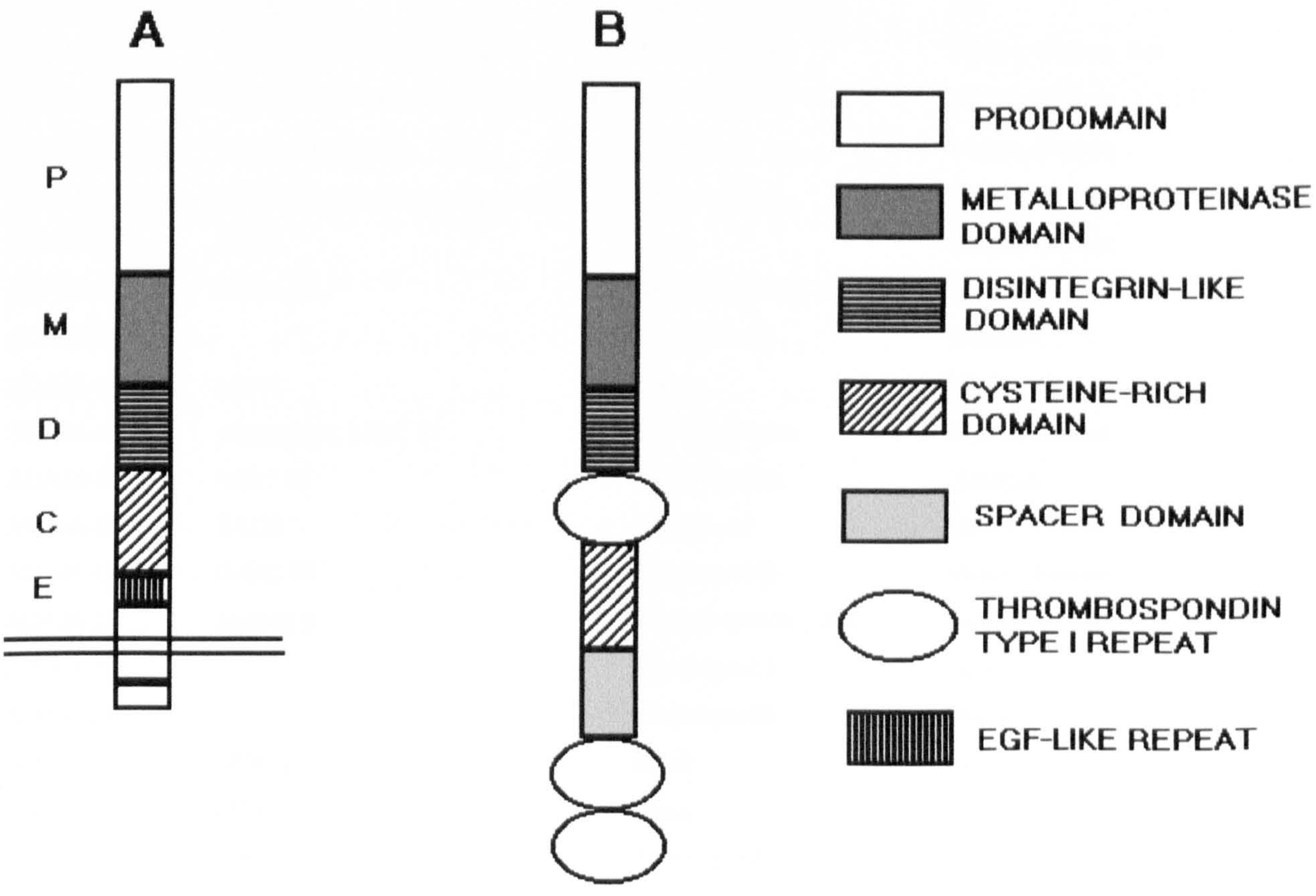


Fig. 1-5 Domain structure of A: ADAMs and B: ADAMTS.

The prodomain (P) blocks protease activity via the cysteine switch mechanism; the metalloprteinase domain (M) has protease activity; the disintegrin domain (D) has adhesion activity; the cysteine rich domain (C) has been shown to have membrane fusion activity in some membrane bound members; the EGF-like domain (E) and cytoplasmic domain present in membrane anchored and absent in ADAMTS. The thrombospondin type I repeat anchors ADAMTS members to the extracellular matrix.

TABLE 1-3 Membrane anchored ADAMs

cDNA	Other Designations	Tissue Distribution	Species
ADAM-1[a]	PH-30 α; Fertilin α	Testis predomniant	Mouse, Human, Rat
ADAM-2	PH-30 β; Fertilin β	Testis specific	Mouse, Human, Rat
ADAM-3	Cyritestin; tMDC I	Testis specific	Mouse, Human, Rat
ADAM-4	tMDC V	Testis predominant	Mouse, Rat
ADAM-5	tMDC II	Testis specific	Mouse
ADAM-6	tMDC IV	Testis predominant	Rat
ADAM-7	Epididymal Apical Protein I	Testis specific	Mouse, Human, Rat
ADAM-8[a]	Macrophage Surface antigen (MS2)	Macrophage	Mouse, Human
ADAM-9[a]	MDC 9; meltrin γ	Ubiquitous	Mouse, Human
ADAM-10[a]	MADM; kuzbanian in Drosophila	Brain, spleen	Mouse, Human
ADAM-11	MDC	Brain	Mouse, Human
ADAM-12[a]	Meltrin α	Skeletal muscle, bone	Mouse, Human
ADAM-13[a]		Neural Crest	Xenopus
ADAM-14	adm-1	Sperm	*C. elegans*
ADAM-15[a]	Metargidin; MDC 15	Cartilage, bone	Mouse, Human
ADAM-16[a]	MDC 16	Testis specific	Xenopus
ADAM-17[a]	TACE	Ubiquitous	Mouse, Human
ADAM-18	tMDC III	Testis specific	Mouse, Human
ADAM-19[a]	Meltrin β	Skeletal muscle, bone	Mouse, Human
ADAM-20[a]		Testis specific	Human
ADAM-21[a]		Testis specific	Human
ADAM-22	MDC 2	Brain	Mouse, Human
ADAM-23	MDC 3	Brain	Mouse, Human
ADAM-24[a]	Testase-1	Testis specific	Mouse
ADAM-25[a]	Testase-2	Testis specific	Mouse
ADAM-26[a]	Testase-3	Testis specific	Mouse
ADAM-27		Testis specific	
ADAM-28[a]	eMDC II		Human
ADAM-29	svph1	Testis specific	Human
ADAM-30[a]	svph4	Testis specific	Human

[a] Contain the metalloproteinase active site sequence HEXGHXXGXXHD

1.4.3.1 Proteinase Activities of ADAMs

Among the 30 known ADAM genes, 17 have a metalloproteinase active site sequence, HEXGHXXGXXHD, and are predicted to be catalytically active (Table 1-3). These ADAMs also contain a potential cysteine switch mechanism, and thus their proteinase domains may be activated by removal of their prodomains. This has recently been shown in the case of ADAM-12 (Loechel et. al., 1999). The physiological substrates for only 4 of these ADAMs have been elucidated; ADAM-9, -10, -17 and -19 have been shown to be involved in protein ectodomain shedding.

Kuzbanian (ADAM-10) has been found to release a soluble form of the Notch ligand (Qi *et. al.* 1999) which is a cell surface receptor which regulates cell fate determination in neurogenesis. More recently Dallas *et al.*, (1999) have shown colocalization of the mammalian orthologue of kuzbanian, ADAM-10, and Notch receptors in osteoblasts and osteocytes at locations of active bone formation. Thus ADAM 10 may be involved in cell-fate determination of osteoblast progenitor cells, possibly during skeletal development and normal bone remodeling. ADAM-10 has also been purified from bovine brain and shown to be capable of degrading myelin basic protein (Chantry *et al.*, 1989) and more recently, Millichip *et al.* (1998) has shown that ADAM-10 purified from bovine kidney has type IV collagenase activity. Whether myelin basic protein and type IV collagenase are true physiological substrates for ADAM-10 is not clear.

ADAM-17 (also called TNF-α converting enzyme, TACE) has been shown by direct biochemical assay to catalyze the release of the membrane anchored form of TNF-α to its soluble form (Moss *et al.*, 1997; Black *et al.*, 1997). Further Peschon *et al.* (1998) showed that TACE-knockout mice were unable to shed TNF-α and were also unable to shed embryonic TGF-α resulting in embryonic lethality in most fetuses. TACE-knockout mice were also unable to shed the TNF receptor, the adhesion molecule L-selectin or amyloid precursor protein (APP), suggesting that TACE has multiple substrates. It has also been shown that ADAM-10 is able to shed TNF-α (Lunn *et al.*, 1997).

ADAM-9 (MDC9) has been reported to shed the heparin-binding EGF-like growth factor (Izumi *et al.*, 1998). Both the membrane-anchored and soluble form of this growth factor are active but the soluble form can act on cells distant from the site of its release.

The remaining 13 proteinases remain 'orphan proteinases', lacking an identified endogenous substrate. Recent biochemical work has shown that the metalloproteinase domain ADAM -12 (meltrin α) is catalytically active. Loechel *et al.* (1998) used the trapping mechanism of α_2-macroglobulin to assay for protease activity of wild-type and mutant ADAM 12 proteins expressed in a COS cell transfection system.

1.4.3.2 Cell -adhesion activity

The disintegrin-like domain of ADAMs shares with PIII snake venom metalloproteinases (PIII SVMPs) an amino acid binding loop which protrudes from the core structure. In PIII SVMPs this binding loop consists of 13 amino acids with a possible integrin binding sequence at its tip consisting of a tripeptide along with an adjacent carboxy-terminal cysteine residue. ADAMs share with their PIII SVMP couterparts the extra cysteine carboxy-terminal to the presumed active site tripeptide, but the sequence of the predicted 14 amino acid binding loop is a great deal more degenerate among the ADAMs (Wolfsberg and White, 1996). The diverse sequences among the ADAMs could reflect that not all ADAMs serve as ligands for integrins. A specific interaction between the ADAM disintegrin-like domain and a cellular receptor has been demonstrated for three members of the ADAMs family.

Almeida *et al.* (1995) demonstrated a specific interaction between the disintegrin domain of ADAM-2 (fertilin β) present on sperm and the α6β1 integrin present on either mouse oocytes or mouse embryonal F9 cells. In sperm-egg or sperm-cultured cell binding assays, a synthetic peptide constituting the integrin binding loop of ADAM-2, was able to inhibit sperm-egg or sperm-cultured cell binding. Use of a monoclonal antibody to the α6 subunit also inhibited binding confirming an association between the disintegrin-like domain and α6β1 integrin.

Another testis-specific sperm surface ADAM (ADAM-3 or cyritestin) has also been implicated in sperm-egg binding (Yuan *et al.*, 1997). An eight-residue peptide from the cyritestin disintegrin loop sequence inhibits sperm-egg adhesion and subsequent fusion.

ADAM-15 (metargidin) is the only member of the ADAM family that has an RGD sequence in its disintegrin-like domain. Zhang *et al.* (1998) used recombinant ADAM-15 expressed in *E. coli* and investigated its binding activity to Chinese Hamster Ovary (CHO) cell lines expressing different recombinant integrins; ADAM-15 specifically interacts with

αvβ3. In addition, Nath *et al.* (1999) identified integrin ligands for ADAM-15 on haemopoietic cells using recombinant ADAM-15 expressed as an Fc fusion protein. ADAM- 15 interacted with the αvβ3 integrin on the monocytic cell line U937 and to the α5β1 integrin on the T cell line, MOLT-4. Iba *et al.* (1999) examined the ability of human ADAM-15 to support tumour cell adhesion. Using an *in vitro* binding assay and recombinant disintegrin-like domain expressed in *E. coli* it was found that the disintegrin-like domain of ADAM-15 supported adhesion to αvβ3-expressing A375 melanoma cells.

Iba *et al.* (1999) have demonstrated that recombinant polypeptides of the cysteine-rich domain of human ADAM-12, but not the disintegrin-like domain supported cell adhesion of a panel of carcinoma cell lines. This interaction could be completely inhibited by pretreatment of the ADAM-12 cysteine-rich domain with heparin leading to the suggestion that ADAM-12 interacts with heparan sulfate chains of the syndecan family of cell surface proteoglycans. More recently Zolkiewska *et al.* (1999) have expressed the disintegrin-like/cysteine-rich domain of mouse ADAM-12 and shown that the recombinant protein supported adhesion of C2C12 myoblasts and NIH 3T3 fibroblasts.

1.4.3.3. Cell-fusion activity

A potential role for members of the ADAM family in cellular fusion was raised by the observation that ADAM-1 posesses a potential fusion peptide consisting of 23 amino acids within the cysteine rich domain that is similar to the potential fusion sequence of rubella virus (Blobel *et al.*, 1992). In addition ADAMS-9, -11 and -12 also possess a potential fusion peptide (Wolfsberg and White, 1996). In support of this expression of antisense RNA to ADAM-12 in the myoblast cell line, C2, suppressed cellular fusion in this cell line and overexpression of ADAM-12 facilitated fusion (Hiromasa *et al.*, 1995).

Studies by Inoue *et al.* (1997) demonstrated expression of ADAM-9, -12, -15 and -19 in osteoblasts, but the expression of ADAM-12 and -19 was not detected in osteoclasts, suggesting that ADAM-12 is not involved in osteoclasts formation. Studies have demonstrated, by *in situ* PCR, that ADAM-12 is expressed in osteoclasts and that addition of antisense oligodeoxynucleotides to bone marrow cultures prevents osteoclast formation (Abe *et al.*, 1999). Although the evidence suggests that ADAM-12 is involved in cellular fusion a definitive role has not been proven.

1.4.4 Cysteine Proteinases

The lysosomal cysteine proteinases, which include the cathepsins B, L, K and S, function at an acidic pH and have been reported to degrade major bone matrix components such as type I collagen *in vitro* (Kirschke *et al.*, 1982; Burleigh *el al.*, 1974). Localization of cathepsins B, L and K in osteoclasts has been demonstrated by immunohistochemical staining (Sasaki and Ueno-Matsuda, 1993; Goto *et al.*, 1993; Littlewood-Evans *et al.*, 1997) suggesting that these enzymes are being exported to the subosteoclastic resorption zone. Further studies have provided direct evidence for participation of cathepsins during bone resorption by the fact that specific cathepsin inhibitors block bone resorption both *in vitro* and *in vivo* (Delaisse *et al.*, 1984, 1987; Hill *et al.*, 1994a) and prevent collagen degradation within the subosteoclastic resorption zone (Everts *et al.*, 1988,1992).

1.4.4.1 Cathepsin B and L

Cathepsin B is the most abundant cysteine proteinase and is expressed as a 36 kDa proform, that is stable at alkaline pH and is converted in the lysosomes to an active 28 kDa form. It is capable of cleaving type I collagen in the N-terminal telopeptide region resulting in depolymerization of the cross linked collagen fibrils (Burleigh, 1977). Cathepsin B is also capable of cleaving collagen types II, IX and XI within the non-helical regions (Maciewicz *et al.*, 1991).

Cathepsin L is synthesized as a proenzyme and processed to an active form of 25 kDa. It has a similar specificity for collagen degradation as cathepsin B although it has been shown to be 20 times more active (Maciewicz, 1987). Kakegawa *et al.* (1993) showed that a specific inhibitor of cathepsin L completely inhibited pit formation by rat osteoclasts whereas no inhibition was observed with a specific inhibitor of cathepsin B. In contrast, specific inhibitors of cathepsin L do not inhibit human osteoclast resorption *in vitro* (James *et al.*, 2001).

1.4.4.3 Cathepsin K

The cDNA encoding cathepsin K was originally cloned from rabbit osteoclasts and designated OC2 (Tezuka *et al.*, 1994a) and has since been isolated from human cDNA libraries independently by several groups (Bromme and Okamoto, 1995; Inaoka *et al.*,

1995; Shi *et al.*, 1995). Bossard *et al.* (1996) demonstrated that cathepsin K is a highly active cysteine proteinase that is capable of hydrolyzing extracellular matrix proteins at pH 5.5. *In situ* hybridization studies demostrated that significantly higher levels of cathepsin K are expressed in osteoclasts than cathepsins B, L, and S (Drake *et al.*, 1996) implicating cathepsin K as having a key role in dissolution of bone matrix during normal bone remodelling and in pathological processes such as osteoporosis and osteoarthritis. The view that cathepsin K plays a pivotal role in bone resorption was strengthened by the identification of mutations in the cathepsin K gene which are linked to pycnodysostosis, a hereditary bone disorder characterized by impaired osteoclast function in bone resorption (Ho *et al.*, 1999). Furthermore, mice with a targeted disruption of cathepsin K exhibit an osteopetrotic phenotype with increased trabecular bone density and retention of cancellous bone in the shafts of long bones (Saftig *et al.*, 1998; Gowen *et al.*, 1999). Garnero *et al.* (1998) showed the collagenolytic activity of cathepsin K is directed both outside the helical region and also at sites inside the helical region. Thus the activity of cathepsin K is sufficient to completely degrade insoluble collagen of cortical bone.

1.5 IN VITRO BONE RESORPTION ASSAYS

In vitro assays for bone resorption have been developed over the last three decades which have increased our understanding of the role of molecules and local and systemic factors in the modulation of bone resorption.

1.5.1 Bone organ cultures

Bone organ cultures were developed by Raisz (1965) for fetal long bones and subsequently adapted to a variety of bone explants including mouse neonatal calvariae (Reynolds and Dingle, 1970) and 17-day-old fetal metatarsal explants (Scheven *et al.*, 1986a). Resorption in these assays is detected by measuring the release of ^{45}Ca as an indicator of mineralized bone resorption or [^{3}H]-proline as an indicator of organic matrix dissolution from prelabelled bones. These assays have been useful in the evaluation of the effects of osteotropic factors such as PTH (Raisz, 1965), 1,25-$(OH)_2D_3$ (Vink-van Wijngaarden *et al.*, 1995), PGE_2 (Stewart and Stern, 1986) and TNF-α (Votta and Bertolini, 1995) on bone resorption. They have also enabled investigators to assess the effects of inhibitors such as bisphosphonates (Boonekamp *et al.*, 1986) and MMP inhibitors

(Hill *et al.*, 1995b). Other parameters of bone resorption have been assessed in organ culture assays including cathepsin release (Delaisse *et al.*, 1984) and release of collagenase (Lenaers-Claeys and Vaes, 1979). Bone resorption assessed by the fetal rat long bone assay and the mouse calvarial assay involve both activation of osteoclasts and osteoclast recruitment making it difficult to assess which phase of bone resorption is being targeted. The development of the 17-day-old fetal metatarsal and metacarpal assays have enabled assessment of the effects of agents on osteoclast formation and migration since at this age mature osteoclasts have not yet developed and only osteoclast precursors are present in the periosteum. Upon culture the osteoclast precursors fuse to form mature osteclasts that migrate into the calcified cartilage which is resorbed (Scheven *et al.*, 1986a).

1.5.2 Isolated osteoclast assay

Methods for the isolation of mature osteoclasts from neonatal rat and rabbit long bones were first devised by Chambers and Magnus (1982). The ability of isolated osteoclasts to excavate resorption lacunae or pits on a mineralized matrix such as bovine cortical bone, dentine or ivory was developed as the basis of a bioassay by Chambers *et al.* (1985a). The assay involved disaggregation of the osteoclasts from the endosteal surface of neonatal rat femurs and tibias. The bone cell suspension was allowed to adhere for only 20 minutes before washing off nonadherent cells producing what approximated to a purified population of osteoclasts although contaminating cells were present. The purity of the osteoclast preparation was demonstrated by their lack of response to osteotropic factors such as PTH (McSheehy and Chambers, 1986), 1,25-$(OH)_2D_3$ (McSheehy and Chambers, 1987), IL-1 and TNF (Thomson *et al.*, 1986, 1987). Subsequently, osteoclasts from a range of sources have been used including 6-day-old mouse, embryonic chick (Oursler *et al.*, 1991a), fetal human (Murrills *et al.*, 1989) and human osteoclastoma (Chambers *et al.*, 1985b). The main disadvantages of this assay are that the osteoclast preparations are impure and the isolated osteoclasts are relatively short lived.

1.5.3 Osteoclast formation assays

Cellular mechanisms controlling differentiation of osteoclasts from haemopoeitic stem cells and commited osteoclast precursor cell populations have been studied using a variety of *in*

vitro bioassays. These assays have provided information on the effects of various osteotropic hormones and local factors on osteoclast formation.

1.5.3.1 Bone marrow cultures

The generation of MNCs with characteristics of osteoclasts was first demonstrated by Testa *et al.* (1981) from long term feline bone marrow culture. Subsequent studies applied this culture system to other animal models including rabbit (Fuller and Chambers, 1989a) and human (MacDonald *et al.*, 1987). The MNCs formed in experiments using feline and rabbit bone marrow possessed many features characteristic of osteoclasts, including TRAP staining, CT receptors and responsivenes to osteotropic hormones such as 1,25-$(OH)_2D_3$ and PTH. In human bone marrow cultures the MNCs formed did not bind calcitonin and were unable to form resorption pits on bone slices, leading to the conclusion that they may have been macrophage-polykaryons (Flanagan *et al.*, 1992). Sarma and Flanagan, (1996) demonstrated that addition of M-CSF to human bone marrow cultures induced large numbers of MNCs that were CT receptor positive and that resorbed bone thus implicating M-CSF as being of critical importance in the formation of human osteoclasts *in vitro*.

Takahashi *et al.* (1988a) developed an assay for generation of numerous TRAP and CT receptor positive MNCs from mouse bone marrow cultured in the presence of 1,25-$(OH)_2D_3$ in which the MNCs formed within 8 days and had the ability to form pits on dentine slices. The effects of various osteotropic and local factors on osteoclast formation have been assessed using the mouse bone marrow culture system. Akatsu *et al.* (1989a,b) demonstrated that PGE_2 and PTH-related protein (PTH-rP) could stimulate osteoclast formation as well as IL-1 (Akatsu *et al.*, 1991).

1.5.3.2 Coculture of hemopoietic cells with fetal bone rudiments

Osteoclast formation from hemopoietic stem cell populations was first demonstrated by Burger *et al.* (1982) in which bone marrow or embryonic liver was cocultured with periosteum-stripped fetal mouse long bones which are devoid of osteoclast precursors. Osteoclasts formed in these cultures that were present in the zone of calcified cartilage.

This assay was refined by Scheven *et al.* (1986b) by fractionating mouse bone marrow into stem cell populations by equilibrium density centrifugation and fluoescence-activated cell sorting (FACS) which were then cocultured with stripped long bones. In this

system osteoclast formation increased with increasing stem cell purity although the culture time required for osteoclast formation increased.

Subsequent studies demonstrated that osteoclasts could be generated in experiments using a clonal pluripotent hemopoietic stem cell line, FDCP-mix A_4, cocultured with fetal bone rudiments (Hagenaars *et al.*, 1991).

1.5.5.3 Coculture of hemopoietic cells with osteoblasts

Takahashi *et al.* (1988b) developed a bioassay in which MNCs were generated in cocultures of mouse spleen cells and mouse calvarial osteoblasts in the presence of 1,25-$(OH)_2D_3$. These osteoclast-like MNCs possessed all the characteristics of osteoclasts including calcitonin receptors and the ability to form resorption pits on dentine slices. Subsequent experiments demonstrated that calvarial osteoblasts could be replaced by the bone marrow-derived, preadipocyte cell lines, MC3T3-G2/PA6 and ST2. These cell lines could support osteoclast formation in this bioassay in the presence of 1,25-$(OH)_2D_3$ and glucocorticoids such as dexamethasone (Udagawa *et al.*, 1989). Chambers *et al.* (1993) established stromal cell lines from a transgenic mouse in which the interferon-inducible major histocompatibility complex promoter drives the simian virus 40 (SV40) temperature sensitive immortalizing gene (*ts*A58). These immortalized cell lines were capable of supporting osteoclast formation in coculture with spleen cells. These cell lines also expressed alkaline phosphatase indicative of an osteoblastic phenotype. Matsumoto *et al.* (1995) also established a bone marrow derived cell line, TM8, from an SV40 large T antigen transgenic mouse which expressed an osteoblastic phenotype and could support osteoclast formation.

Several cell lines of hemopoietic origin have been shown to have osteoclastic potential when cocultured with osteoblasts. Hattersley and Chambers, (1989b) demonstrated that the FDCP mix A_4 multipotential cell line was capable of generating osteoclasts when cocultured with bone marrow stromal cells. Cell lines of hemopoietic origin have been derived from the *H-2K^{b}tsA58* transgenic mouse (Chambers *et al.*, 1993) which when cocultured with stromal cell lines on dentine slices in the presence of 1,25-$(OH)_2D_3$ resulted in extensive resorption.

Bioassays analyzing osteoclastic lineage have been developed using a two-step coculture system of osteoblasts with hemopoietic cells collected from colonies formed in

semi-solid medium in the presence of CSFs (Hattersley and Chambers, 1989b; Hattersley *et al.*, 1991; Takahashi *et al.*, 1991; Kerby *et al.*, 1992). Takahashi *et al.* (1991) demonstrated using this system that marrow cells recovered from semosolid cultures supported by M-CSF (M-CFU) formed more TRAP-positive and CTR-positive MNCs when cocultured with osteoblasts than did GM-CSF, G-CSF or IL-3. In a similar investigation using spleen cells as a source of hemopoietic stem cells Kerby *et al.* (1992) demonstrated that multilineage colonies had a greater potential to form osteoclasts as assessed by pit formation than single lineage colonies.

1.6 PROGRAMMED CELL DEATH

Programmed cell death (PCD) or apoptosis is the process whereby cells activate an intrinsic death program and kill themselves. PCD occurs in normal tissue turnover (Wyllie *et al.*, 1980b), embryogenesis (Hammar and Mottet, 1971), metamorphosis (Decker, 1976), and during tumour growth and regression (Kerr *et al.*, 1972). Cells that die in this manner normally undergo a characteristic set of morphological and biochemical changes (Fig 1-6): they appear shrunken, with extensive membrane blebbing and nuclear fragmentation (Wyllie *et al.*, 1980b). PCD is an energy dependent process requiring ATP and occurs under normal physiological conditions. Necrosis occurs in circumstances of wide departure from physiological conditions and is not energy dependent. The terminal stages in PCD are characterized by the cell fragmenting into a number of membrane bound vessicles called apoptotic bodies. These apoptotic bodies contain a variety of cytoplasmic organelles such as mitochondria which appear intact and of normal appearance and some also contain nuclear fragments. These membrane bound vesicles are eventually phagocytosed by macrophages without the involvement of an inflammatory reaction (Wyllie *et al.*, 1980b). In contrast, cells undergoing necrosis swell and rupture, releasing their contents and thereby eliciting an inflammatory response (Wyllie *et al.*, 1980b). Ultrastructurally, the cytoplasm of cells undergoing PCD becomes condensed though cellular organelles remain intact.

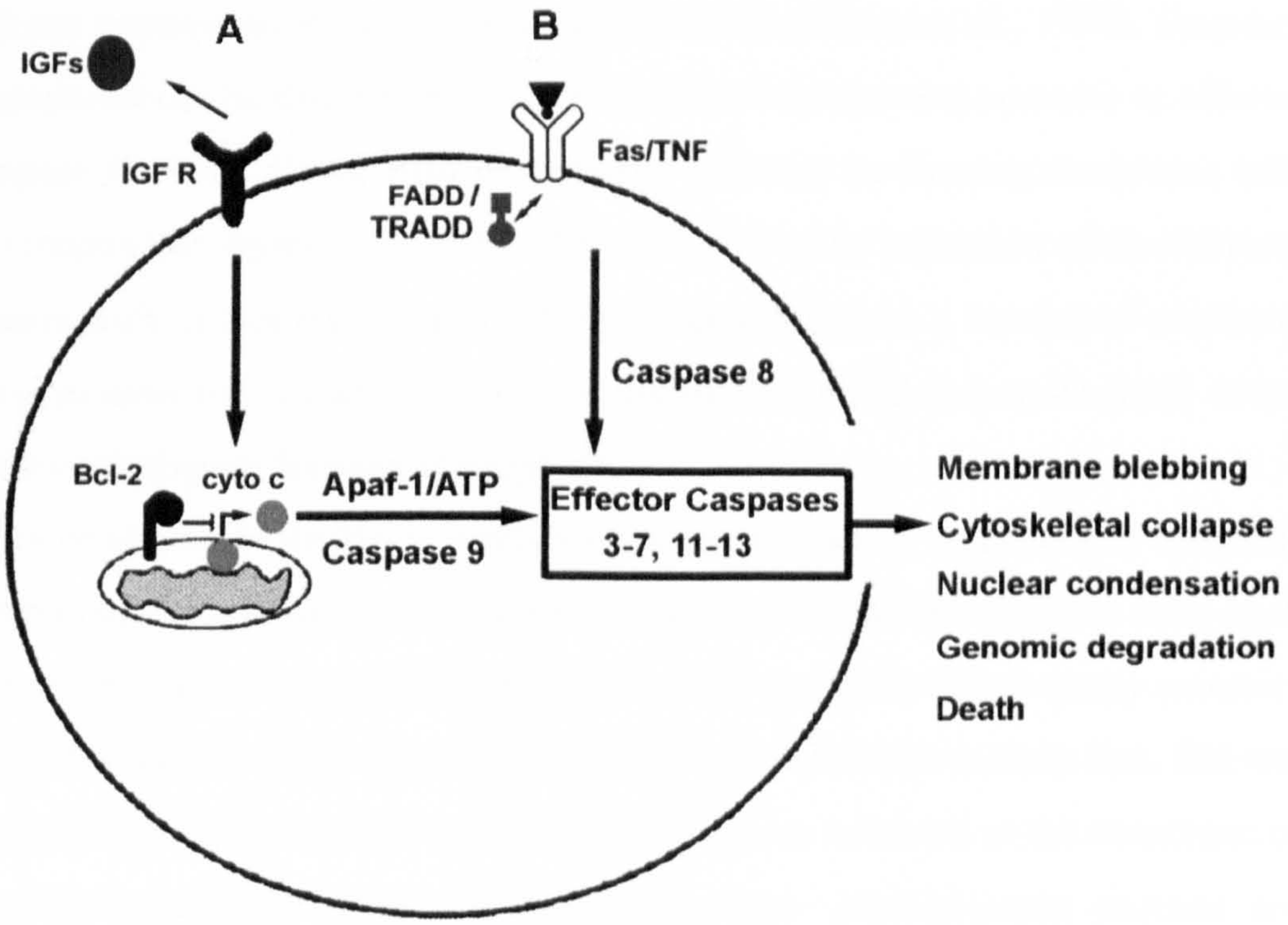


Fig. 1-6 General overview of apoptosis (from Dragovich et al. 1997).

Mitochondrial stress caused by growth factor withdrawal (A) induces release of cytochrome *c* into the cytoplasm which interacts with Apaf-1, ATP and procaspase-9 resulting in the auto-catalytic activation of caspase-9 and subsequent activation of effector caspases-3 and -7. Release of cytochrome *c* is regulated by Bcl-2 family members. In receptor-mediated apoptosis (B) interaction of death factor such as TNF-α with TNFR1 or Fas ligand with Fas initiates recruitment of TNF-receptor associated death domain (TRADD) or Fas associated death domain (FADD) and procaspase-8 molecules with the subsequent catalytic activation of caspase-8.

Ultrastructural changes in the nucleus are characterized by chromatin condensation and margination at the nuclear membrane followed by nuclear fragmentation. Chromatin condesation in PCD is associated with the activation of an endogenous deoxyribonuclease which excises nucleosome chains from nuclear chromatin. This results in an oligoonucleosomal ladder-type fragmentation such that the degraded DNA will form a 200-bp ladder pattern when separated by gel electrophoresis (Wyllie *et al.*, 1980b).

The morphological features observed in cells undergoing apoptosis arise from the activities of a family of cysteine proteinases called caspases which form part of an evolutionarily conserved mechanism for triggering apoptosis. Fourteen caspases have been

identified and are synthesized as proenzymes of 30-50 kDa (Nunez *et al.*, 1998). Caspases involved in apoptosis can be divided into two subfamilies: initiator and executor or effector caspases. Caspase 8, 9 and 10 are initiator caspases activated by forming complexes with death factor receptors or Apaf-1 (Fig. 1-6). This is followed by activation of downstream effector caspases such as caspase 3 and 6. Effector caspases cleave a number of structural and regulatory proteins such as lamins and cytokeratins (Stroh and Schultz-Osthoff, 1998) leading to the morphological features of apoptosis.

Proteins belonging to the Bcl-2 family are major intracellular regulators of apoptosis that are evolutionarily conserved. In mammals 15 members of the Bcl-2 family have been identified that either suppress or promote apoptosis. Anti-apoptotic Bcl-2 family members include the proteins Bcl-2 and Bcl-X_L whilst the proapoptotic proteins include Bax, Bid and Bad (Adams and Cory, 1998). Proteins of the Bcl-2 family are localized to the membrane of mitochondria, the endoplasmic reticulum and the nucleus. Anti-apoptotic proteins are believed to inhibit apoptosis by interfering with the release of cytochrome *c* from the mitochondrial membrane (Fig. 1-6). The ratio of pro-apoptotic:anti-apoptotic proteins is believed to determine a cells susceptibility to apoptosis (Merry and Korsmeyer, 1997)

1.6.1 Apoptosis induced by TNF-α

Tumour necrosis factor-α (TNF-α) and its receptors (TNFR1 and TNFR2) are the prototype members of two superfamilies which play an important role in regulating many biological processes (Lotz *et al.*, 1996). Over 20 members of the TNF receptor superfamily and 9 cognate ligands of the ligand superfamily have been identified thus far in mammalian cells (Orlinick and Chao, 1998). Of the members of the TNF ligand family, three are potent death factors inducing apoptosis by binding to their cognate receptors thereby signalling the cell death pathway (Nagata, 1997). TNF-α has been shown to induce apoptosis in several cell lines including the human leukemia cell lines HL-60 and U937 (Greenblatt and Elias, 1992) and the murine fibrosarcoma cell lines L292 and WEH1 (Fulton and Chong, 1992). Other death factors include Fas ligand (FasL) also called CD95L or Apo1L. This ligand induces apoptosis by binding to its receptor, Fas, also called CD95 or Apo1. A third member known as TRAIL (TNF related apoptosis inducing ligand) also called Apo2L has been identified that induces apoptosis in tumorigenic or transformed cells but not in normal cells (Wiley *et al.*, 1995). TRAIL can interact with four distinct receptors: DR4, DR5,

DcR1 and DcR2. Both DR4 and DR5 are type I transmembrane proteins that contain cytoplasmic death domains and upon ligation with TRAIL, mediate apoptosis (Pan *et al.*, 1997). In contrast neither DcR1 nor DcR2 mediates apoptosis upon ligation with TRAIL. Because they lack the ability to directly signal cell death DcR1 and DcR2 have been hypothesized as being protective factors, either by acting as "decoy" receptors (Sheridan *et al.*, 1997) or by transduction of an antiapoptotic pathway (Degli-Esposti *et al.*, 1997).

TNF-α induces apoptosis by binding to its cognate receptor TNFR1 or TNFR2. Although both TNFR1 and TNFR2 can transduce the signal for apoptosis, TNFR1 is responsible for these signals in most cases (Vandenabeele *et al.*, 1995). TNFR1 contains a homologous cytoplasmic sequence (about 80 amino acids) which has been designated a "death domain" (Targalia *et al.*, 1993). This sequence, which is also found in the receptors which interact with FasL and TRAIL, enables death receptors to engage the cells apoptotic machinery (Ashkenazi, 1998) and activation of caspases (Fig. 1-6).

1.6.2 Ceramide Mediated Apoptosis

Ceramide is a sphingosine based lipid second messenger molecule that has been shown to mediate TNF stimulated apoptosis in the human myeloid leukemia cell line U937 (Dressler *et al.*, 1992). It has been shown in this cell line that activation of the TNFR1 receptor promotes rapid breakdown of the phospholipid, sphingomyelin, increasing intracellular free ceramide levels within seconds. Gulbins *et al.* 1995 have demonstrated that the sphingomyelin pathway is engaged with similar kinetics following specific ligation of Fas in Jurkat T cells. Activation of TNF receptors stimulates neutral and acidic sphingomyelinases (N- and A-SMases) which catalyze the hydrolysis of sphingomyelin to ceramide and phosphorylcholine. Definitive evidence for the critical role of A-SMase and ceramide in initiating apoptotic signaling was provided by studies using genetic models of A-SMase deficieny. Lymphoblasts from patients with Niemann-Pick disease, an inherited disorder of A-SMase, and A-SMase knockout mice show defects, but not total loss, of the apoptotic response (Santana *et al.*, 1996).

Ceramide has been shown to be an activator of the stress activated protein kinase/c-JUN N-terminal kinases (SAPK/JNK) pathway (Verheij, 1996). This pathway is activated by many stress stimuli such as irradiation and reactive oxygen species any of which may lead to apoptosis (Verheij, 1996).

1.6.3 Apoptosis in Bone Cells

1.6.3.1 Osteoblasts

Apoptosis has been observed in the murine clonal osteoblast cell line MC3T3-E1 transfected with the human T-cell leukemia virus type I (HTLV-I) *tax* gene (Kitajima *et al.*, 1996a). HTLV-I infection causes adult T-cell leukemia (ATL) which is characterized by hypercalcemia-associated bone lysis (Bunn *et al.*, 1983). In studies of HTLV-I transfected MC3T3-EI cells, these cells underwent apoptosis in response to TNF-α and this was associated with activation of the NF-κB signalling pathway (Kitajima *et al.*, 1996b). It has therefore been suggested that the induction of apoptosis by TNF-α and the activation of NF-κB in HTLV-I *tax*expressing osteoblasts may be involved in the pathogenesis of bone lesions in patients with HTLV-I related disease. More recently, Jilka *et al.*, (1998) reported that murine osteoblasts undergo apoptosis after serum withdrawal and after addition of TNF-α.

1.6.4.2 Osteoclasts

Recruitment, differentiation and activity of osteoclasts are tightly controlled by systemic and local factors. Vitamin D_3, prostaglandins, TGF-β, IL-1, IL-6 and TNF-α stimulate osteoclast differentiation and activity via direct or indirect mechanisms. The fate of osteoclasts after bone resorption is largely unknown. A number of factors such as bisphosphonates, vitamin K_2 and glucocorticoids have been shown to induce apoptosis in osteoclasts (Hughes *et al.*, 1995; Kameda *et al.*, 1996; Dempster *et al.*, 1997). It has been reported that estrogen promotes apoptosis of murine osteoclast-like cells mediated by TGF-β in a mixed cell population (Hughes *et al.*, 1996). Thus estrogen may prevent excessive bone loss before and after the menopause by limiting osteoclast lifespan through promotion of apoptosis. It has reported that 17-β-estradiol was able to inhibit osteoclastic bone resorption activity in part by targeting osteoclasts directly to undergo apoptosis through estrogen receptor mediated mechanisms. In this study 17-β-estradiol induced osteoclast apoptosis in a dose- and time-dependent manner (Kameda *et al.*, 1997). Other studies have demonstrated apoptosis of osteoclast precursors in bone marrow cultures in response to elevated levels of nitric oxide (van't Hof and Ralston, 1997) and osteoclast apoptosis following inhibition of vacuolar H^+-ATPase (Okahashi *et al.*, 1997). Furthermore, targeting

the α_v integrin subunit with oligodeoxynucleotides has been shown to induce osteoclast apoptosis (Villanova *et al.*, 1999).

1.7 AIMS

The aims of the current study are firstly to investigate the effects of cytokines and bone matrix derived growth factors on osteoblast survival *in vitro*. Secondly to investigate the effects of IL-11, serine proteinases and members of the ADAM family on osteoblast and osteoclast function using *in vitro* bioassays.

2. Multiple Extracellular Signals Affect Osteoblast Survival

2.1 INTRODUCTION

Programmed cell death (PCD) has been established as a mechanism for maintaining cellular homeostasis during normal tissue turnover (Wyllie *et al.*, 1980b). Skeletal tissue undergoes remodelling throughout life, influenced by both systemic and mechanical demands. It has been estimated that 65% of osteoblasts present at a remodelling site fail to differentiate into bone lining cells or osteocytes and that these cells die by apoptosis (Jilka *et al.*, 1998). Recent studies highlight the importance of osteoblast apoptosis in bone diseases such as glucocorticoid induced osteoporosis, and the use of bisphosphonates (Plotkin *et al.*, 1999) and estrogen (Gohel *et al.*, 1999) in preventing apoptosis in osteoblasts.

In many models of PCD, cells are induced to die as a result of changes in environmental stimuli (Jacobson *et al.*, 1994; Elis *et al.*, 1991; Duke and Cohen, 1986). In general these studies of PCD suggest that the absence of a "survival factor," such as a particular hormone or growth factor, will induce a cell to initiate its own cell death. Skeletal cells and other cells within the bone microenvironment, synthesize a variety of growth factors (GFs) and cytokines (Canalis *et al.*, 1993b). The extracellular matrix of bone has been shown to be an abundant source of several polypeptide factors, most notably TGF-β (Seyedin *et al.*, 1986), IGF's-I and -II, PDGF, and acidic and basic FGFs (Haushka *et al.*, 1986). When released and presented to responsive cells during phases of bone resorption, these GFs influence bone remodelling in conjunction with IL-1 and TNF-α, cytokines produced mainly by bone marrow mononuclear cells. IGFs are important skeletal GFs, not only because of their abundance in bone but also because they have important actions on bone cell function and are expressed by skeletal cells (Delaney *et al.*, 1994). These various GFs and cytokines influence the differentiated function of osteoblasts and bone resorption by interacting with cell surface receptors present on osteoblasts. These effects are critical for the formation of new bone and for the maintenace of bone matrix.

Based on the importance of GFs and cytokines in bone remodelling their effects on osteoblast survival and apoptosis *in vitro* have been investigated.

2.2 MATERIALS & METHODS

2.2.1 Materials

Human recombinant TNF-α was purchased from R&D systems (Abingdon, Oxon, UK). αIR-3, a monoclonal antibody to the type-I IGF receptor was obtained from Oncogene Sciences (Cambridge, UK). Terminal deoxynucleotidyl transferase, biotinylated dUTP, streptavidin fluorescein, trypsin, dispase and bacterial collagenase A were purchased from Boehringer Mannheim (Lewes, East Sussex, UK). Human recombinant IGFs-I and II, insulin from bovine pancreas, bFGF, EGF, PDGF, TGF- β, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and all cell culture reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK). M-CSF, G-CSF and GM-CSF, interferon-gamma (IFN-γ) and murine LIF were purchased from British Biotechnology Ltd. (Oxford, UK) 1,25-$(OH)_2D_3$ was a gift from Roche (Welwyn Garden City, UK). Deoxy[5-^{3}H]-cytidine, specific activity 888 GBq/mmol, was purchased from Amersham International plc (Little Chalfont, Buckinghamshire, U.K). Litters of 1-day-old CD-1 mice were purchased from Charles Rivers (Kent, UK).

2.2.2 Methods

2.2.2.1 Preparation of osteoblasts from neonatal mouse calvariae

Calvarial osteoblasts were prepared and characterized as previously described (Heath *et al.*, 1984). Briefly, neonatal mouse calvariae were dissected free from adherent soft tissue, washed in Ca^{2+} and Mg^{2+} free Tyrode's solution (Appendix 1) and sequentially digested with 1 mg/ml trypsin (10 min), 2 mg/ml dispase (30 min) and 2 mg/ml bacterial collagenase A (3 x 30 min). Cells released by the last two collagenase digestions were washed and grown in alpha modification of minimum essential medium (α-MEM) containing 10% FBS and antibiotics for 4 days prior to use. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. For survival assays, osteoblasts were plated in 100 μl of serum free CMRL-1066 medium containing 10^{-3} M thymidine to block cell proliferation, with or without added cytokines. Osteoblasts were characterized for alkaline phosphatase by incubating the cells for 45 min at room temperature in 0.1M Tris-HCl pH 8.3 containing 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml fast red TR

salt. Intracellular accumulation of cyclic AMP in response to PTH was measured by enzyme immunoassay (Amersham, UK).

2.2.2.2 Cell survival assays

MTT assay. Mitochondrial function was assayed by the ability of viable cells to convert soluble MTT into an insoluble dark-blue formazan reaction product (Mosmann, 1983). In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well of a 96 well plate was measured photometrically as previously described (Mosmann, 1983). MTT was dissolved in phosphate buffered saline (PBS, Appendix 1) at a concentration of 5 mg/ml and sterilized by passage through a 0.22 μm filter. This stock solution was added (one part to 10 parts medium) to each well of a 96 well tissue culture plate, and the plate was incubated at 37°C for 4 h. Acid-isopropanol (400 μl of 10 M HCl in 100 ml isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few min at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader (Labsystems EIA reader) at a wavelength of 570 nm-630 nm. A standard curve was set up using 200 to 50,000 cells/well and the absorbance was directly proportional to the number of cells over this range. The % survival was defined as $[(\text{experimental}_{\text{absorbance}} - \text{blank}_{\text{absorbance}})/ \text{control}_{\text{absorbance}}-\text{blank}_{\text{absorbance}})] \times 100$ where the $\text{control}_{\text{absorbance}}$ was the value obtained for 10,000 cells/well which is the number plated at the start of the experiment and the $\text{blank}_{\text{absorbance}}$ was the value obtained in wells containing medium and MTT without cells.

Plasma membrane permeability. Plasma membrane permeability was assessed using the membrane-impermeant DNA dye, ethidium homodimer, which labels dead cells. Live cells were labelled using the membrane permeant dye calcein AM. (Molecular Probes Inc., Leiden, Netherlands) Calcein AM is a non fluorescent dye which is converted into the fluorescent dye calcein by intracellular esterases, and is retained only in cells with an intact plasma membrane. The concentrated dyes were prepared according to the manufacturers instructions, and were added to unwashed cells in culture to final concentrations of 4 μM for ethidium homodimer and 2 μM for calcein AM.

2.2.2.3 Cell Proliferation assay

Primary osteoblasts were plated at a density of 1 x 10^4 cells/well of a 96 well plate and cultured for 48 h in CMRL-1066 medium with GFs or FCS in the presence or absence of non-radioactive thymidine (10^{-3} M). The cells were pulsed for the last 6 h with 1 μCi [^{3}H]-cytidine and DNA associated radioactivity was performed at the end of the experiment by fixing the cells with 5% TCA at 4°C for 10 min, washing in PBS and the cells were detached with trypsin-EDTA solution (0.5%:0.02%).

2.2.2.4 Analysis of DNA fragmentation by agarose gel electrophoresis

DNA fragmentation was analyzed by agarose gel electrophoresis. Primary mouse osteoblasts were grown to confluence in 75 cm^2 flasks and then cultured in serum free CMRL-1066 medium with or without TNF-α. Adherent cells were lysed with 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA in 0.3% SDS and incubated with proteinase K (500 μg/ml) at 55°C for 15 h. Samples were extracted with an equal volume of phenol:chloroform, and the total DNA contained in the aqueous phase was precipitated with a 1/10 volume of sodium acetate (3 M, pH 6.6) and a 2.5 volume of ethanol at -80°C for 15 h. DNA pellets were obtained by centrifugation at 13,000 x *g* (Labofuge 400R, Heraeus Instruments, Germany) for 15 min and resuspended in 50 μl of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Samples were then treated with 10 U/ml of DNAse-free RNAse for 1 h at 37°C. Electrophoresis was performed on a 1% agarose gel in 1X Tris-Acetate-EDTA (Appendix 1) at 50 V for 1.5 h in the presence of 0.5 μg/ml of ethidium bromide. DNA bands were visualized on a UV transiluminator (UVP inc., UK).

2.2.2.5 Terminal Deoxynucleotidyl Transferase-mediated dUTP-Biotin Nick End Labeling

DNA cleavage was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) reaction, as described by Gavrieli *et al.* 1992. Primary mouse osteoblasts were cultured in labtech chamber slides (Life Technologies, Paisley, Scotland) in serum free CMRL-1066 medium, containing thymidine (10^{-3} M) and with or without GFs. After 48 h in culture, the cells were fixed in 4% paraformaldehyde for 10 min, washed in 10 mM Tris-HCl, pH 8.0, and then permeabilized in 0.1% Triton X-100 in 10 mM Tris-HCl, pH 8.0, for 5 min. After washing in 10 mM Tris-

HCl, pH 8.0, the cells were preincubated for 10 min at room temperature in the reaction buffer for terminal deoxynucleotidyl transferase (200 mM potassium cacodylate, 0.22 mg/ml BSA, 25 mM Tris-HCl, pH 6.6). After 10 min the preincubation buffer was removed and reaction mixture containing 500 U/ml terminal deoxynucleotidyl transferase, 2.5 mM $CoCl_2$, and 40 μM biotinylated dUTP added. After 60 min at 37°C, the reaction was terminated by the addition of 300 mM NaCl and 30 mM sodium citrate. After 25 min at room temperature cells were washed with PBS, and incubated with streptavidin fluorescein for 60 min at room temperature in the dark. After extensive washing in PBS, the cells were examined with a Leitz Laborlux fluorescence microscope.

2.2.2.6 Electron Microscopy

Primary mouse osteoblasts were plated at 2 x 10^5 cells/ml on glass cover slips that had been previously coated with poly-L-lysine. Cells were cultured for 48 h in serum free CMRL-1066 medium with or without TNF-α. After 48 h cells were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.3) at 4°C for 4 h. After washing in PBS, the cells were postfixed in 1% osmium tetroxide in phosphate buffer at 4°C for 30 min. The cells were then dehydrated in ascending grades of ethyl alcohol and embedded in taab resin. After removing the glass cover slip thin sections were cut on a Reichert ultramicrotome. The sections were stained with a saturated solution of uranyl acetate and a 4% solution of lead citrate and examined using a Hitachi H7000 electron microscope (Tokyo, Japan).

2.2.2.7 Statistical analysis

Data are presented as the mean ± SEM of six to twelve cultures per group. Each experiment was repeated three times. Differences between control and treatment groups were determined by the Mann-Whitney U test.

2.3 RESULTS

2.3.1 Characterization of murine osteoblasts

Histochemical staining of unstimulated primary cultures for alkaline phosphatase was strongly positive; 95.3 ± 3.1% of cells from six separate bone cell preparations exhibited positive staining (Fig. 2-1). The intracellular accumulation of cAMP levels in response to PTH was determined; treatment with PTH (10^{-8} M) for 10 min resulted in cAMP levels of 12.3 ± 1.2 pmole/ml, compared with control levels of less than 0.125 pmole/ml.

2.3.2 Survival of primary osteoblasts in culture

When osteoblasts were cultured in serum-free and insulin-free CMRL 1066 medium containing thymidine (10^{-3} M) about 67% of cells survived after 24 h whilst only about 28% of cells survived in this medium after 48 h (Table 2-1). However, when the osteoblasts were cultured with 2% FCS in the presence of thymidine (10^{-3} M), which blocks cell proliferation (Murgo *et al.*, 1980) and therefore permits the assessment of factors on cell survival, there was a 100% survival of the cells at both time intervals.

As can be seen in Table 2-1 bFGF, IGF-I, IGF-II or a concentration of insulin (10^{-6} M) high enough to activate IGF-I receptors increased the number of surviving osteoblasts after a 48 h culture period but had no effect on their survival after 24 h. In contrast, the immunoregulatory cytokine TNF-α induced cell death in the 24 and 48 h osteoblast cultures (Table 2-1). A variety of other GFs and osteotropic factors that were tested had no effect on either osteoblast survival or cell death, namely PDGF, EGF, TGF-β, interleukins-1, -3, -6, -11, LIF, IFNγ, M-CSF, GM-CSF, G-CSF and the osteotropic hormones PTH and $1,25\text{-}(OH)_2D_3$.

TABLE 2-1. Effects of cytokines and growth factors on the survival of primary mouse osteoblasts under serum free culture conditions.

Treatment	Conc. (M)	% Survival	
		24 h	48 h
Control		65 ± 4	28 ± 3
FCS	2%	98 ± 5[a]	102 ± 6[a]
Insulin	10^{-6}	63 ± 4	65 ± 2[a]
IGF-I	10^{-7}	71 ± 5	72 ± 3[a]
IGF-II	10^{-7}	68 ± 2	64 ± 4[a]
bFGF	20 ng/ml	62 ± 2	57 ± 3[a]
PDGF	10^{-9}	65 ± 3	32 ± 3
GM-CSF	10^{-10}	67 ± 3	26 ± 2
G-CSF	10^{-10}	60 ± 4	31 ± 4
M-CSF	10^{-10}	62 ± 3	27 ± 1
IL1α	10^{-11}	68 ± 4	36 ± 6
IL3	10^{-10}	62 ± 3	33 ± 3
LIF	100U/ml	64 ± 2	23 ± 2
IL-6	10^{-9}	59 ± 5	21 ± 1
IL-11	10^{-9}	67 ± 3	25 ± 4
EGF	10^{-9}	65 ± 3	28 ± 3
TGF-β	10^{-11}	61 ± 2	27 ± 3
TNF-α	10^{-10}	18 ± 2[a]	11 ± 1[a]
IFNγ	10^{-8}	67 ± 3	32 ± 3
PTH	2 U/ml	60 ± 5	31 ± 3
1,25-$(OH)_2D_3$	10^{-8}	69 ± 3	28 ± 1

Mouse osteoblasts were cultured at a cell density of 10,000 cells per well of a 96 well plate in serum free CMRL-1066 medium in the presence of thymidine (10^{-3} M) and the above growth factors. After incubation for 24 and 48 h cell survival was assessed by MTT survival assay. The results are the mean ± SEM of 12 separate cultures. [a] $P < 0.05$ significantly different from the control.

The effects of bFGF, IGF- I, IGF-II and insulin on cell number were due to effects on osteoblast survival, rather than on cell proliferation as the addition of thymidine to the culture medium at a 10^{-3} M concentration effectively blocked the proliferative effects of these factors. As shown in Table 2-2, these factors both increased the number of surviving cells and proportionally decreased the number of dead cells, so that the total numbers of cells in the factor-containing microwells were not statistically different from the numbers in medium alone, thereby confirming that thymidine had effectively blocked DNA synthesis. The addition of thymidine did not induce cell death as the proportion of live/dead cells was similar in its presence/ absence (Table 2-2).

Labelling osteoblast DNA with [^{3}H]-cytidine confirmed that non-radioactive thymidine effectively blocked the effects of FCS and the various GF combinations on osteoblast proliferation (Table 2-3).

TABLE 2-2. Effects of IGF-I, IGF-II, bFGF, and insulin on the numbers of live and dead osteoblasts.

Factors added	Thymidine (10^{-3} M; +/-)	No. of cells after 48 h by phase contrast	
		Live cells	Dead cells
None	-	71 ± 15	509 ± 54
None	+	60 ± 18	486 ± 43
Insulin (10^{-6} M)	+	306 ± 41	278 ± 36
IGF-I (10^{-7} M)	+	351 ± 39	246 ± 46
IGF-II (10^{-7} M)	+	329 ± 46	229 ± 39
bFGF (20 ng/ml)	+	249 ± 37	254 ± 46

Primary mouse osteoblasts were cultured at a density of 1000 cells/well of a 96-well plate in the presence (+) or absence (-) of thymidine (10^{-3} M). Approximately 50-60% of the cells adhered to the wells. After 48 h, the total numbers of live and dead cells were counted by phase contrast microscopy. The results are the mean ± SEM of triplicate cultures.

TABLE 2-3. Effects of non-radioactive thymidine (10^{-3}M) on osteoblast proliferation in the presence of IGF-I, bFGF, and PDGF using [^{3}H]cytidine.

Treatment	Non-radioactive thymidine (+/-)	dpm
Control	+	1987 ± 143
2% FCS	–	8123 ± 436
2% FCS	+	1787 ± 129
IGF-I	–	5329 ± 431
IGF-I	+	1601 ± 97
bFGF	–	4097 ± 231
bFGF	+	1585 ± 198
IGF-I/bFGF/PDGF	–	9754 ± 432
IGF-I/bFGF/PDGF	+	1768 ± 154

Primary mouse osteoblasts were cultured at a density of 10,000 cells/well of a 96-well plate in CMRL medium with and without non-radioactive thymidine for 48 h. IGF-I, bFGF and PDGF were used at 10^{-6}M, 20 ng/ml, and 10^{-9}M respectively. Cells were labelled with [^{3}H]cytidine. The results are the mean ± SEM of quadruplicate cultures.

2.3.3 Mechanism of osteoblast cell death

The morphology of the cells that died by 24 h in the absence of survival factors and in the presence of either TNF-α (10^{-10} M) or a high concentration (10^{-6} M) of the protein kinase inhibitor staurosporine was consistent with their having died by PCD rather than by necrosis. The cells exhibited the typical apoptotic morphology of cell shrinkage and membrane blebbing (Fig. 2-2). A characteristic feature of apoptosis is nuclear fragmentation (Wyllie *et al.*, 1980b), which can be visualized by fluorescence microscopy using the membrane impermeant dye ethidium homodimer and the membrane permeant dye calcein. As shown in Fig. 2-3A cells cultured in the presence of 2% FCS for 48 h contained intact nuclei, which were identical to nuclei of cells that were cultured in the absence of serum for only 2 h. Cells cultured in the absence of serum for 24 h, however, showed extensive nuclear fragmentation (Fig. 2-3B). In many cases these fragments were associated with apoptotic bodies, the small membrane-bound vesicles released by apoptotic cells (Wyllie *et al.*, 1980b).

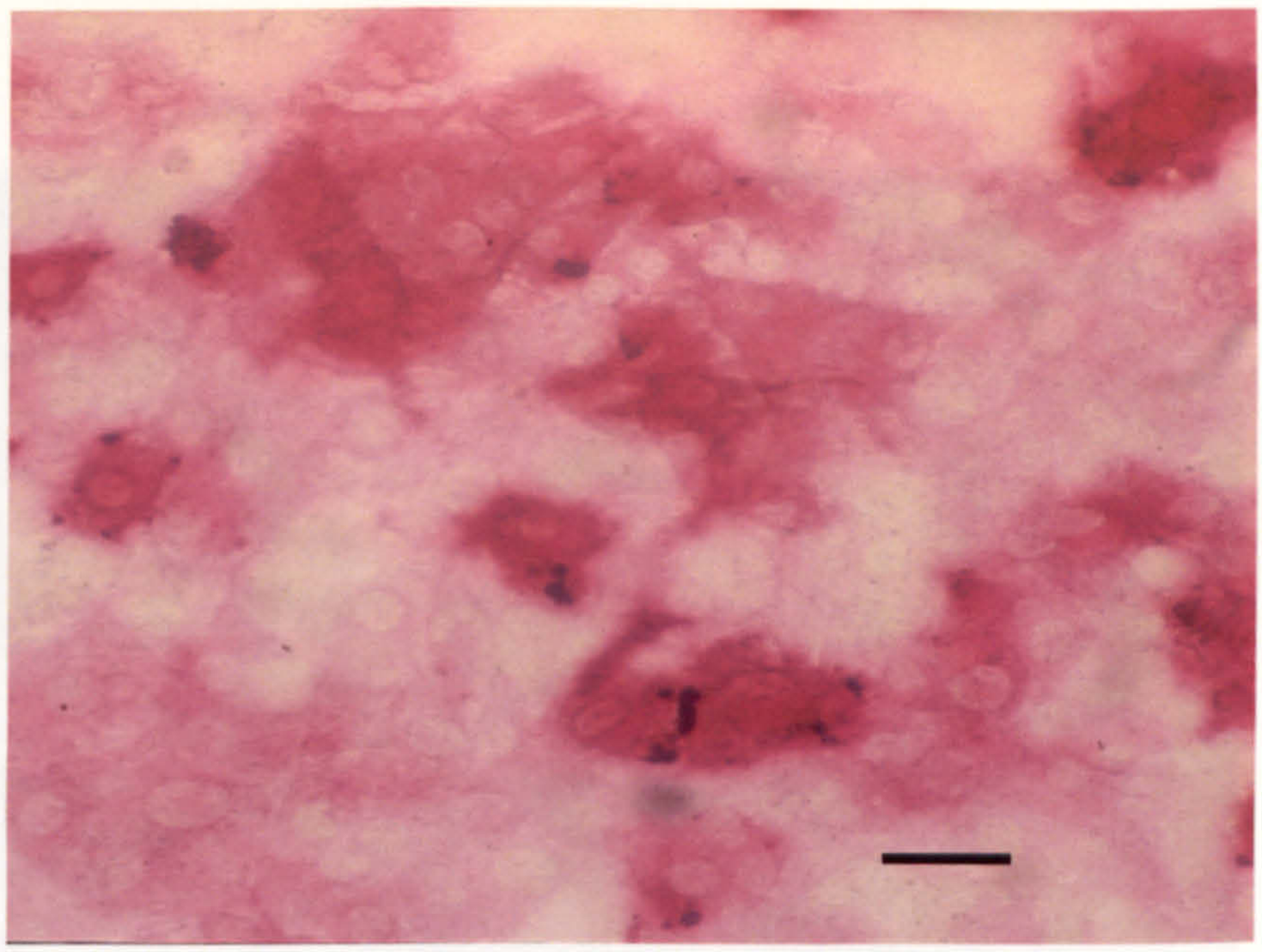


FIG. 2-1. Photomicrograph of primary mouse osteoblasts stained for alkaline phosphatase.
Primary mouse osteoblasts were grown to confluence in MEM + 10% serum and stained for alkaline phosphatase as described in Materials and Methods. Bar = 10 μm. Osteoblasts exhibited strong staining for alkaline phosphatase.

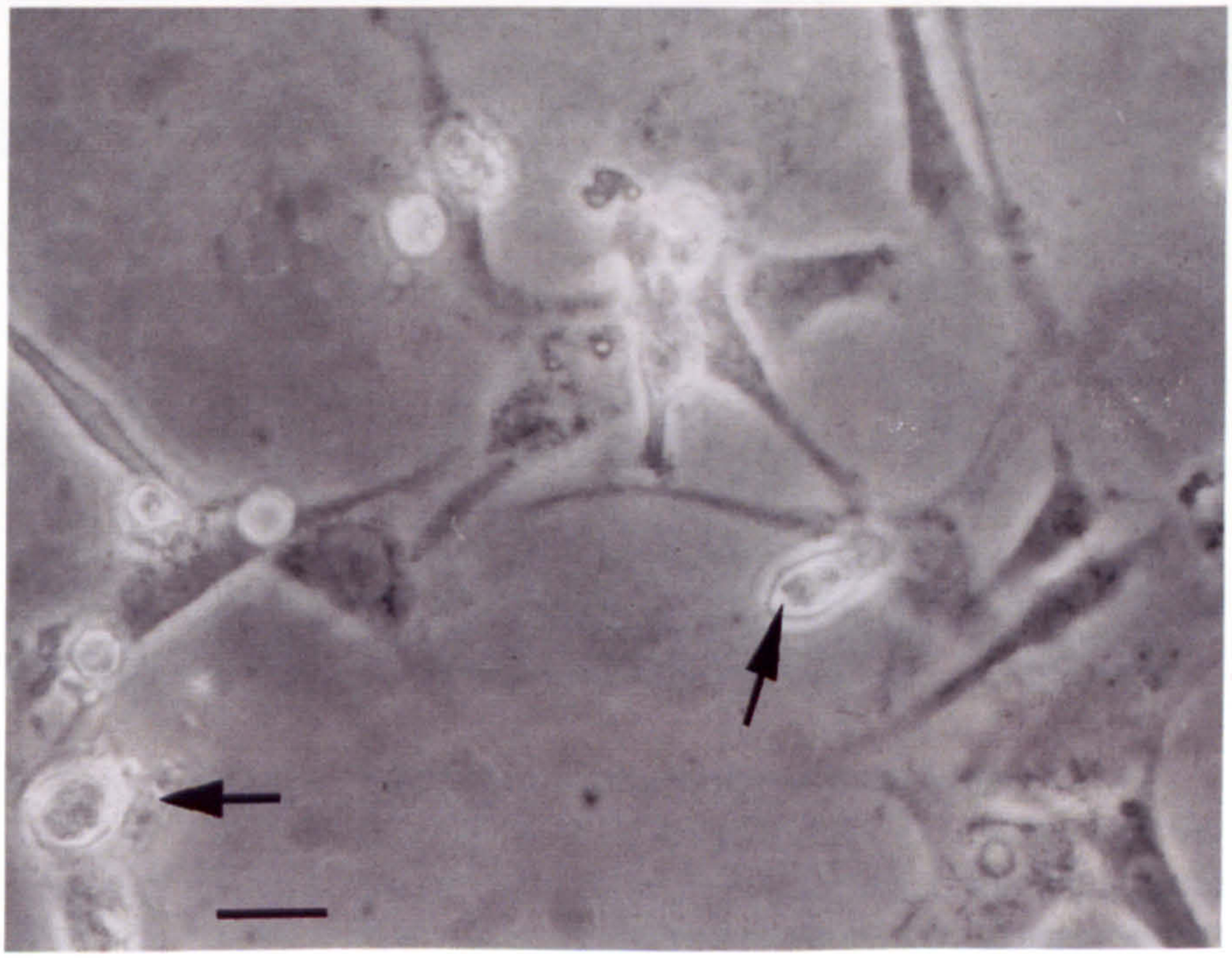


FIG. 2-2. Phase contrast micrograph of osteoblasts.
Osteoblasts were maintained in the absence of serum for 24 h. Arrows show degenerating cells. Bar = 20 μm.

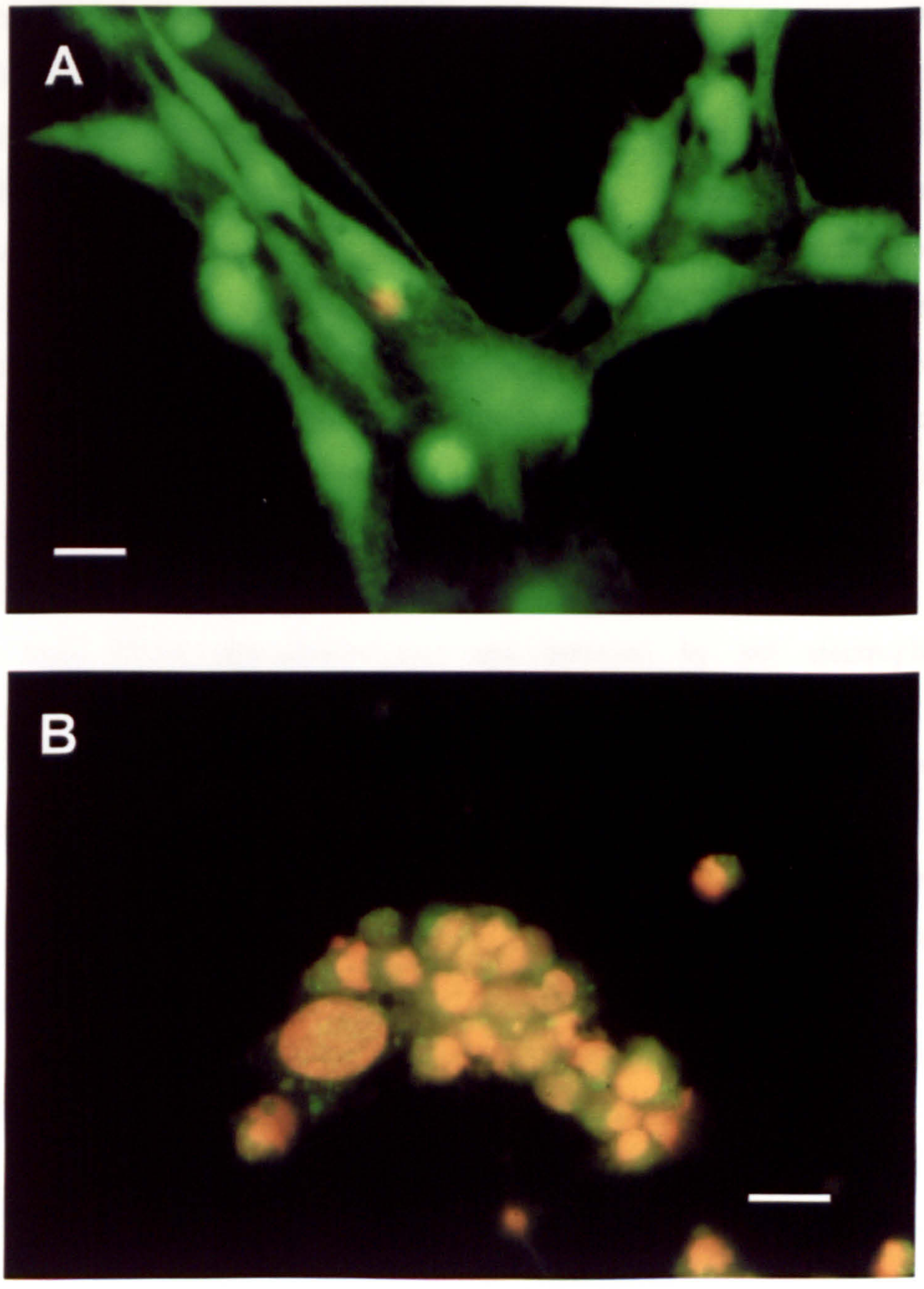


FIG.2-3. Plasma membrane permeability of osteoblasts
Osteoblasts (5000 cells/well) were cultured in Lab-Tek slide chambers in the presence of 2% FCS (A) or in its absence (B) for 48 h. The cells were then stained with ethidium homodimer and calcein and examined by fluorescence microscopy. A, Typical appearance of live cells. B, Typical examples of dead cells. Bar = 10 μm.

Many investigators have reported that endogenous deoxyribonucleases are activated in cells undergoing PCD (Compton, 1992) with DNA being degraded to form 200 bp ladders (Wyllie, 1980a). To assay for DNA degradation, DNA was analyzed by agarose gel electrophoresis. As shown in Fig. 2-4 DNA isolated from osteoblasts cultured in the absence of serum migrated as a single, high molecular weight band (Fig. 2-4, lane 3). On the other hand, in four separate experiments, DNA extracted from osteoblasts that were cultured for 3, 6, 12, or 24 h in the presence of either TNF-α or staurosporine, an agent which induces DNA degradation in many cell types, showed no evidence of DNA degradation into oligonucleosome fragments (Fig. 2-4, lanes 1,2 respectively), which is often observed in PCD (Wyllie, 1980a). In contrast, U937 leukemia cells cultured under identical conditions exhibited the characteristic pattern of DNA degradation in response to either TNF-α or staurosporine (Fig. 2-4, lanes 4,and 5) whilst no degradtion was observed when the cells were cultured in serum (Fig. 2-4, lane 6).

Since DNA degradation was not detected by gel electrophoresis, DNA fragmentation in individual cells was demonstrated by specific labelling of double-strand DNA breaks using terminal deoxyribonucleotidyl transferase, otherwise referred to as the TUNEL method (Gavrieli *et al.*, 1992). As shown in Fig. 2-5A osteoblasts cultured in the presence of 2% FCS for 48 h demonstrated negligible DNA fragmentation by TUNEL. However, TUNEL of osteoblasts cultured with TNF-α for 48 h confirmed that a substantial cell death had taken place in accordance with the results obtained with the MTT assay (Fig. 2-5B). The TUNEL method showed innumerable cells with clear-cut staining indicative of chromatin condensation.

Electron microscopy confirmed that TNF-α induced PCD in osteoblasts, as the chromatin was usually seen to be compacted and segregated into sharply defined masses under the nuclear membrane (Fig. 2-6A), although margination of chromatin was observed in about 50% of these cells (Fig. 2-6A); the cytoplasmic organelles, in particular the mitochondria, remained largely intact. As a basis for comparison a swollen necrotic osteoblast is shown in Fig. 2-6B. In contrast to apoptosis there is a total disruption of the internal structure of the cell undergoing necrosis whilst the normal morphology of the osteoblast is preserved when it is cultured in the presence of IGF-I (Fig. 2-6C).

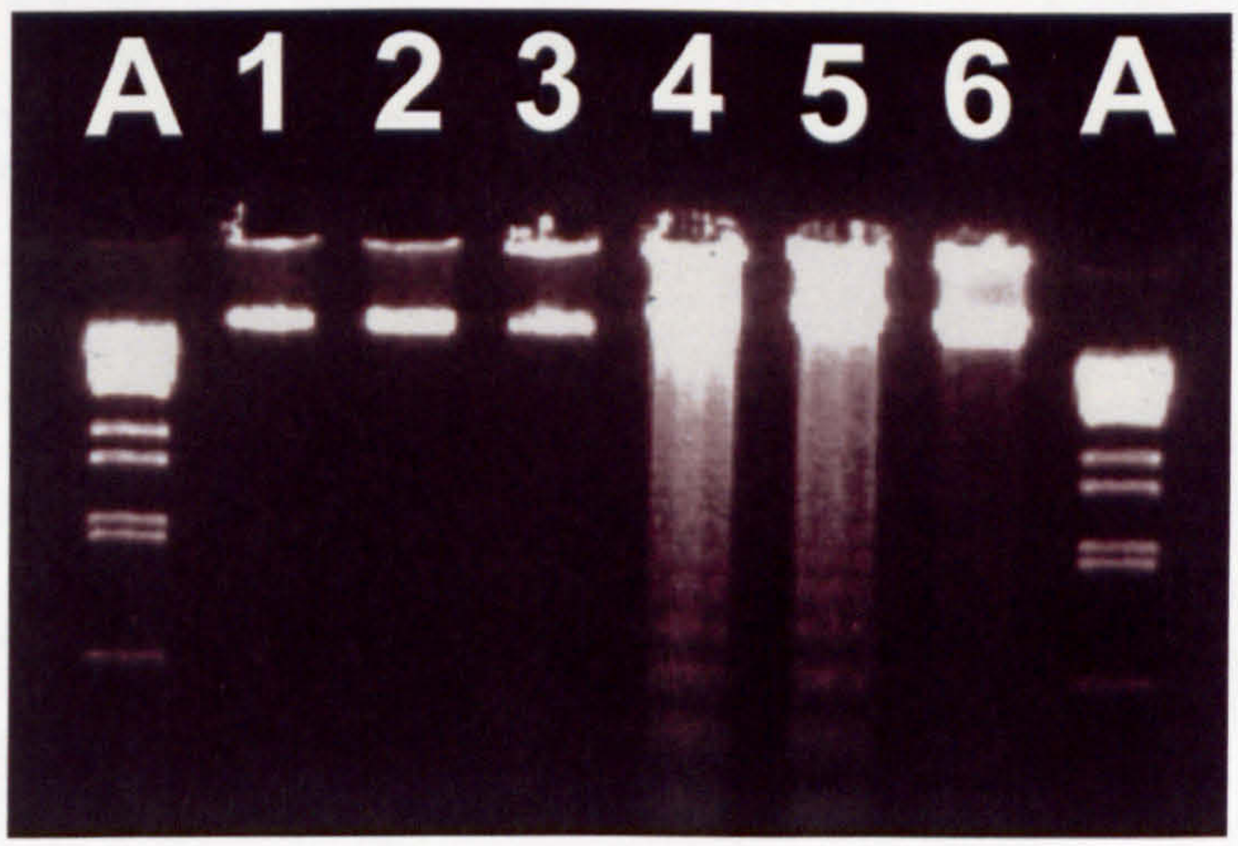
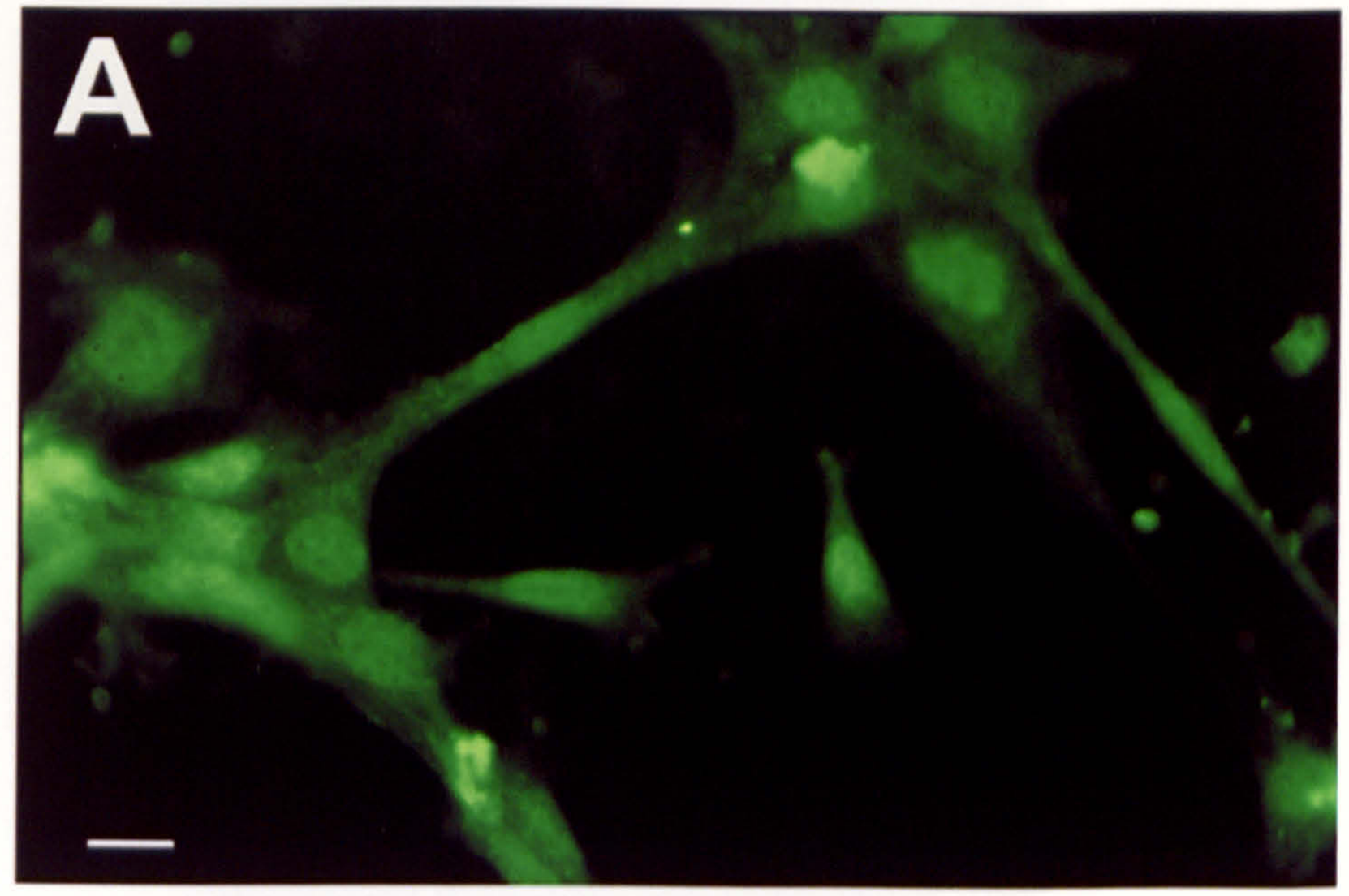


FIG. 2-4. DNA Fragmentation.
Primary murine osteoblasts or U937 leukemia cells were lysed, and cellular DNA was isolated and subjected to gel electrophoresis on a 1% agarose gel. Lane 1, DNA isolated from osteoblasts cultured in serum-free medium containing TNF-α (10^{-10} M) for 24 h. Lane 2, DNA isolated from osteoblasts cultured in serum-free medium containing staurosporine (10^{-6} M) for 24 h. Lane 3, DNA isolated from osteoblasts cultured in the absence of serum. Lanes 4 and 5, U937 cells that were cultured as described for osteoblasts in lanes 1 and 2, respectively. Lane 6, U937 cells cultured with FCS. A, DNA size marker (λ BstEII digest).



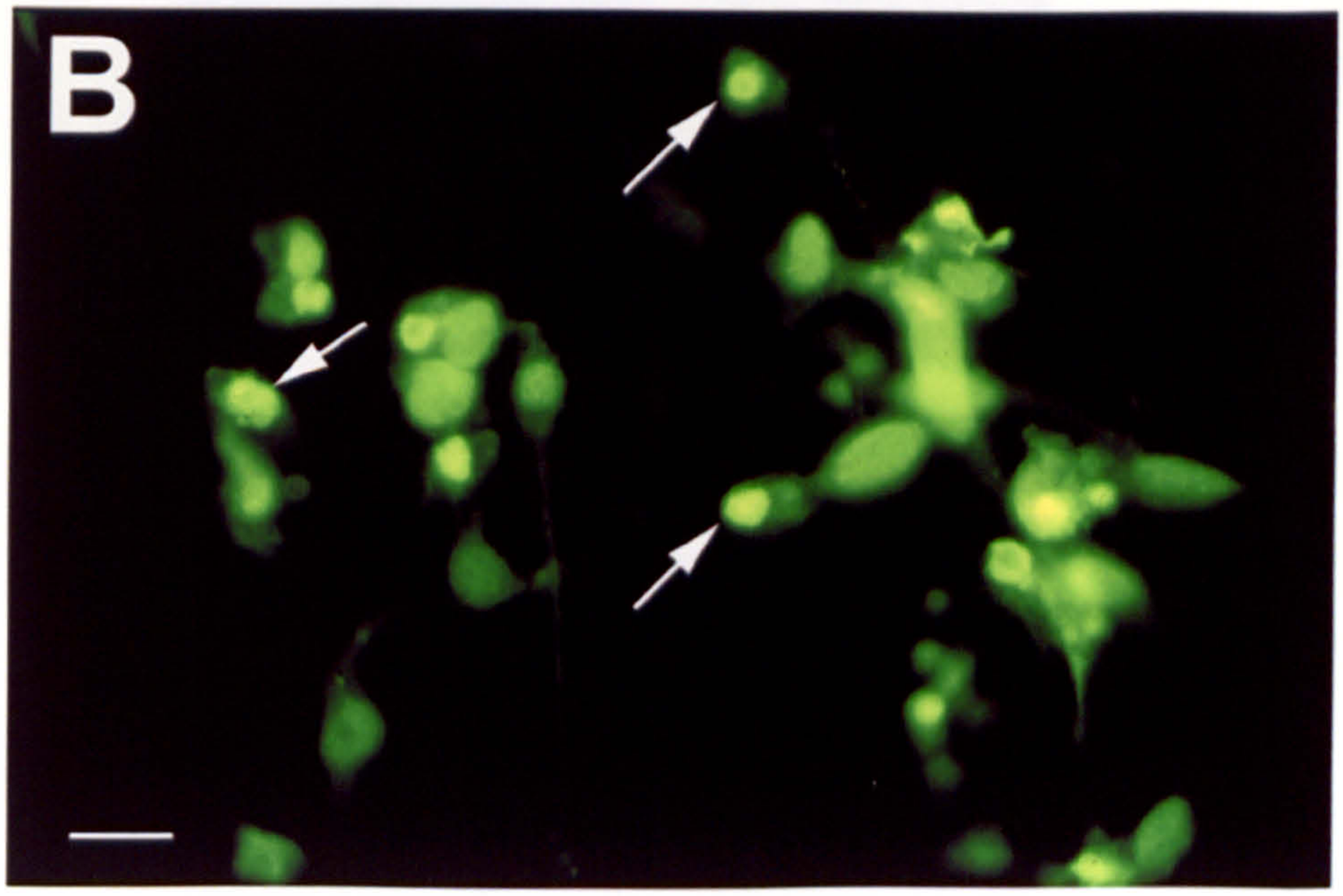


FIG. 2-5. *In situ* DNA labelling by the TUNEL technique.
Mouse osteoblasts were cultured as described in Materials and Methods in the absence (A) or presence (B) of TNF-α (10^{-10} M). After 48 h the cells were fixed, permeabilized, and processed for TUNEL. A, Typical example of osteoblasts showing negligible DNA fragmentation; B, cells cultured in the presence of TNF-α show extensive DNA fragmentation. Bar = 10 μm.

FIG. 2-6. Transmission electron microscopy.

A, After treatment with TNF-α for 6 h, the chromatin is highly condensed against the margins of the nuclei, which contains only an amorphous fibrillar network throughout their interior. Cytoplasmic organelles, in particular mitochondria remain intact. B, After treatment with sodium nitroprusside (2 mmol/L) for 16 h, necrotic cells could be seen which contained swollen nuclei, with few identifiable cytoplasmic organelles. For comparison, a normal osteoblast cultured in the presence of IGF-I for 24 h is shown in C. Bar = 5.7 μm.

Having established that TNF-α induced apoptosis in mouse osteoblasts the effects of graded concentrations of this cytokine on PCD in murine osteoblasts was investigated using the MTT assay. TNF-α dose-dependently (10^{-14}-10^{-9} M) decreased the survival of primary mouse osteoblasts over a 24 h culture period from a level of survival of 67 ± 2.8% at 10^{-14} M down to 18 ± 1.1% at 10^{-9} M (Fig. 2-7).

2.3.4 Effects of insulin, IGF-1, IGF-2 and bFGF on osteoblast survival in vitro

To determine the survival effects of different concentrations of insulin and IGFs on primary osteoblasts, these cells were cultured in CMRL 1066 serum-free medium with thymidine (10^{-3} M) to prevent proliferation, and survival assessed after 48 h with the MTT assay. In the presence of plateau concentrations of insulin, IGF-I, IGF-II or bFGF, about two thirds of the cells survived for 2 days (Fig. 2-8 a,b,c and d respectively). The concentration that promoted half-maximal survival (the ED_{50}) was about 10^{-9} M for IGF-I, and 10^{-8} M for IGF-II as expected if both IGFs promote survival by binding to IGF-I receptors (Sara and Hall, 1990). The ED_{50} for insulin-induced survival was about 10^{-7} M and the ED_{50} for bFGF was about 5 ng/ml.

2.3.5 Effects of multiple factors on osteoblast survival

Because single factors did not promote 100% survival of osteoblasts, the effects of combinations of different GFs on short-term survival were investigated. The combination of insulin, IGF-I or IGF-II, for example, did not promote survival better than insulin, IGF-I or IGF-II alone (table 2-4). In contrast, a combination of a plateau concentration of bFGF with either insulin, IGF-I, or IGF-II produced an additive effect on survival (Fig. 2-8 a,b,c) with a maximum of about 75%. A similar effect was seen when a plateau concentration of IGF-I was used in combination with graded concentrations of bFGF (Fig. 2-8d) Surprisingly, although PDGF did not promote survival on its own, when combined with either insulin, IGF-I, or IGF-II survival was enhanced (Fig. 2-9 a,b,c). The combination of bFGF and PDGF with IGF-I, did not promote survival better than bFGF with IGF-I (table 2-4). A similar situation was found with bFGF/PDGF/IGF-II and bFGF/PDGF/insulin combinations (table 2-4). There was also no additive effect on survival when several of the other GFs such as TGF-β, EGF, IL-1, IL-11 were used in combination with the IGFs or insulin (table 2-4).

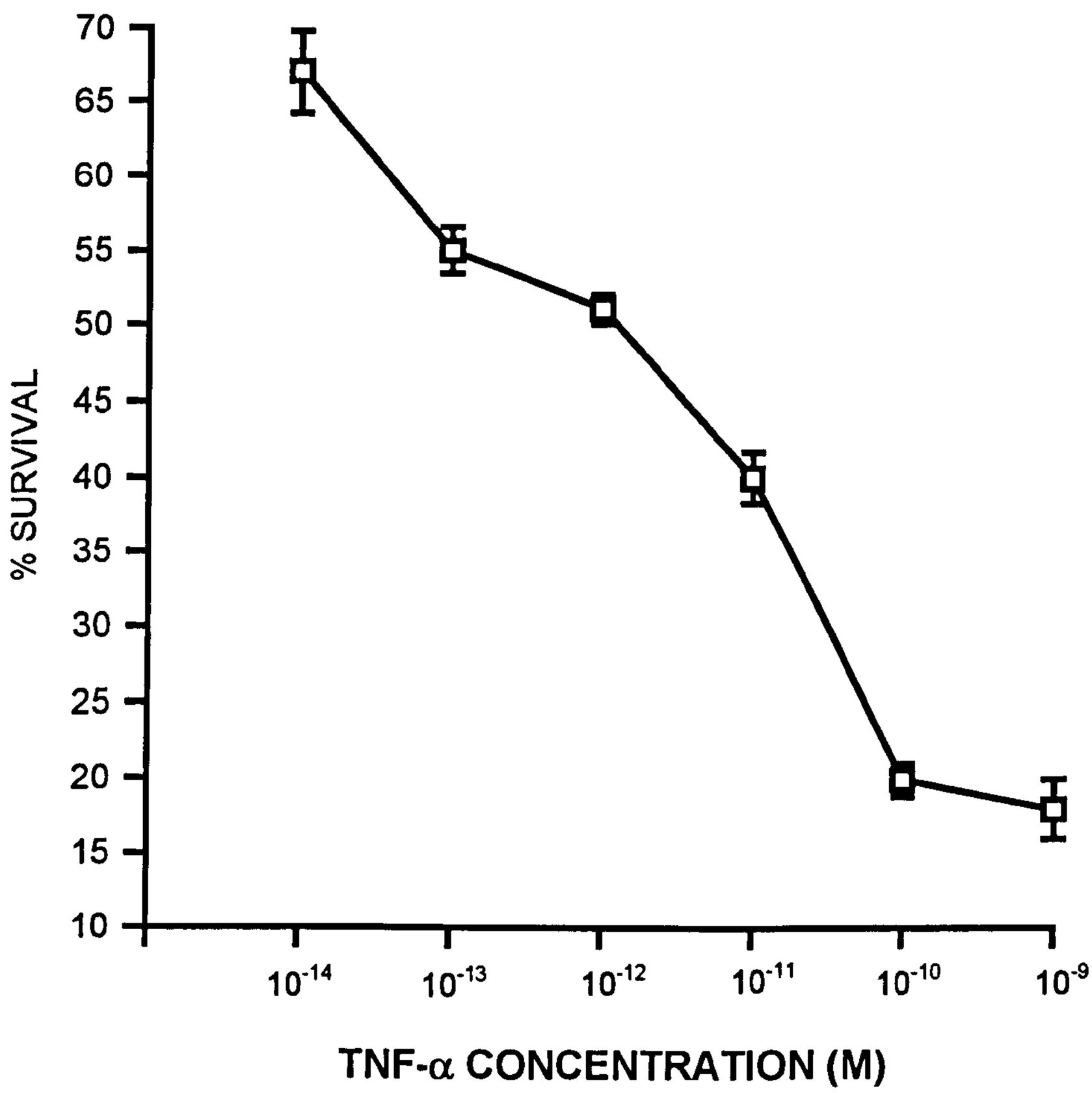


FIG. 2-7. Effect of TNF-α on osteoblast survival *in vitro*.

Mouse osteoblasts were cultured as described in Materials and Methods in the presence of increasing concentrations of TNF-α. Vehicle (CMRL-1066 medium) was used as a control. After 48 h in culture, cell survival was assessed by the MTT cell survival assay. Each point is the mean ± SEM of six wells. TNF-α dose-dependently decreased osteoblast survival.

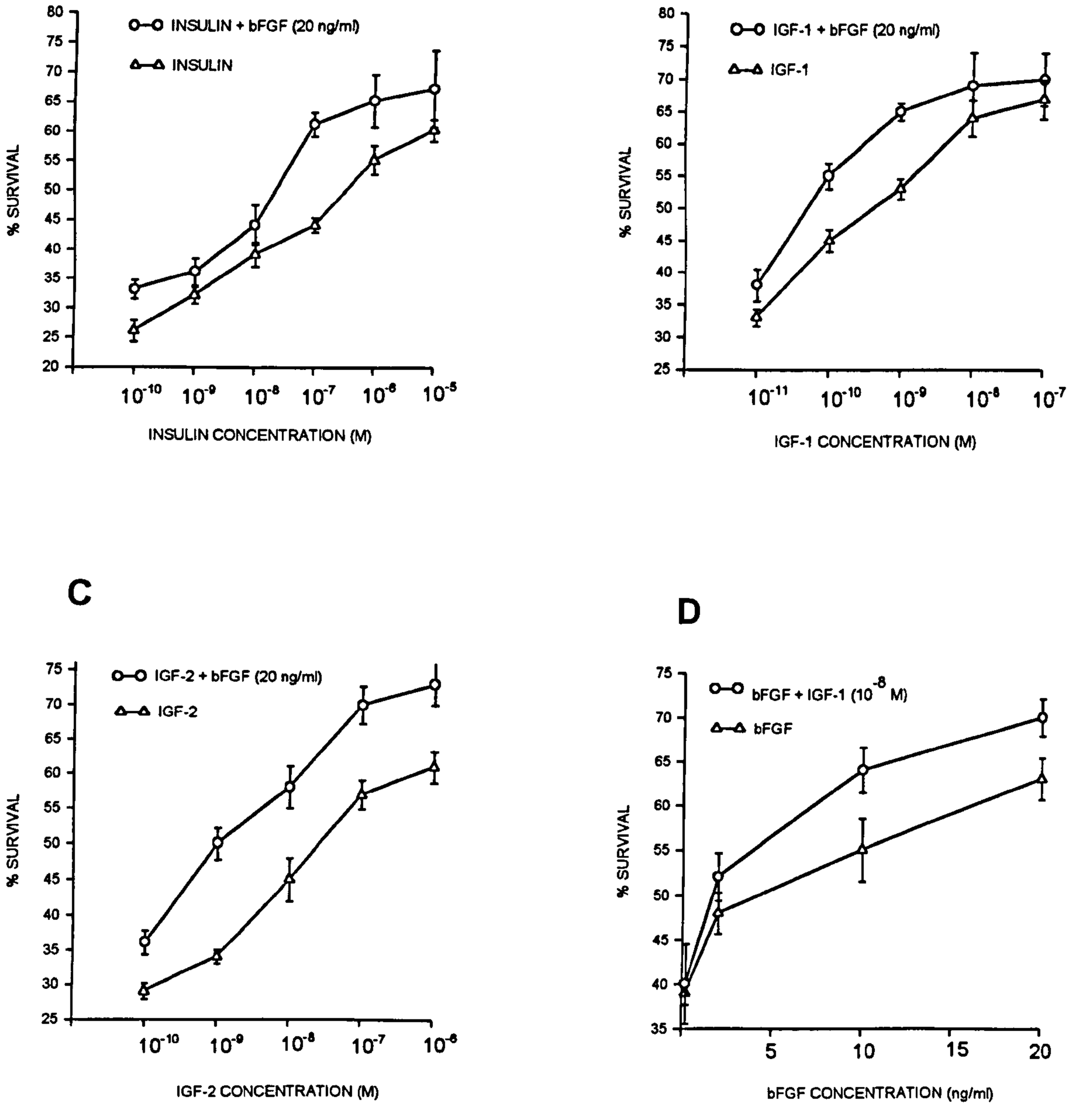


FIG. 2-8. Effects of insulin, IGF-I, IGF-II, and bFGF, alone and in combination, on osteoblast survival *in vitro*.
Mouse osteoblasts were cultured as described in *Materials and Methods* in the presence of increasing concentrations of insulin (a), IGF-1 (b), IGF-2 (c), or bFGF (d). These GFs were also added in combination. CMRL-1066 medium without FCS was used as a control. After 48 h, cell survival was assessed by the MTT cell survival assay. Each point is the mean ± SEM of six wells. The experiment was repeated six times.

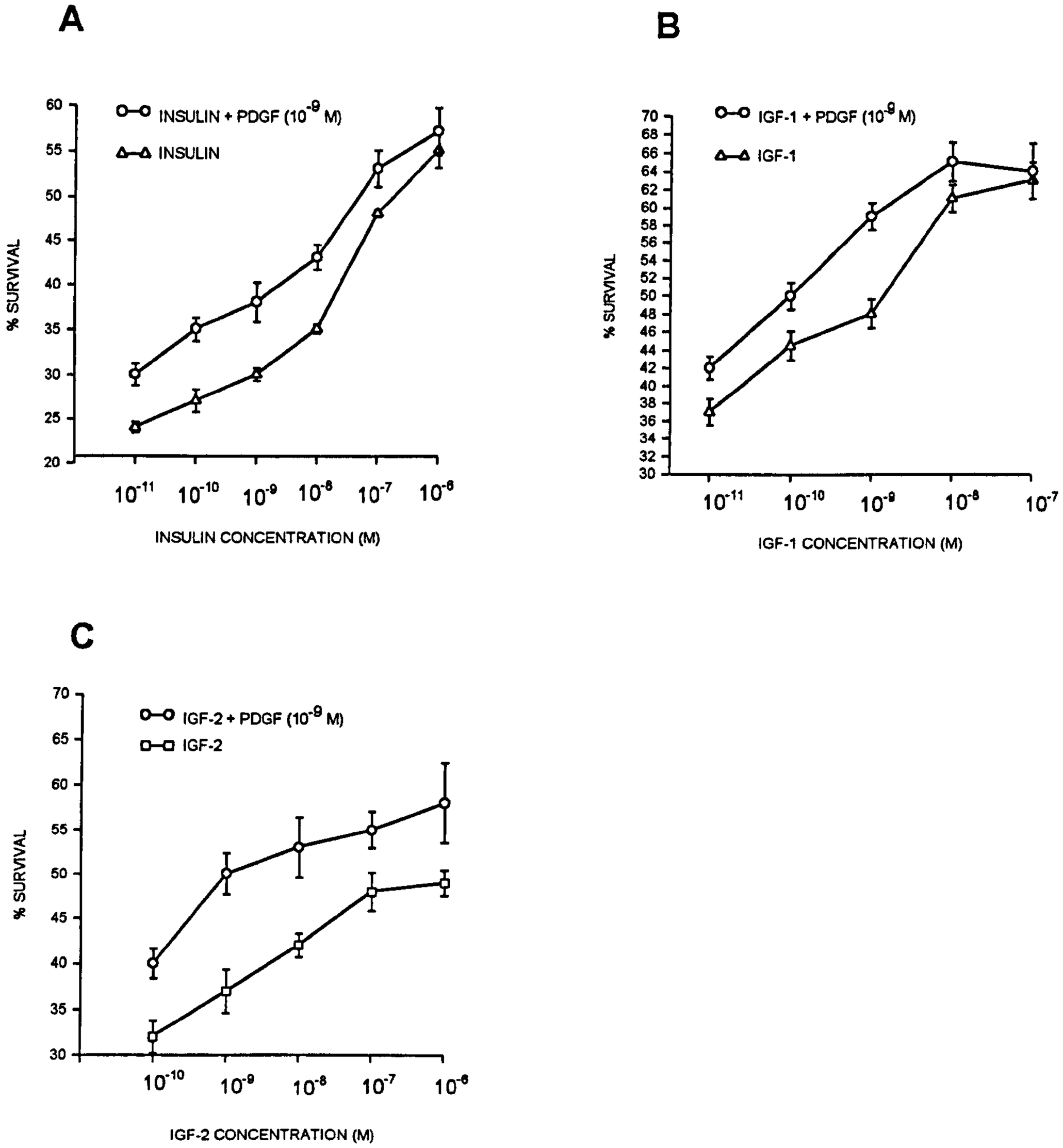


FIG. 2-9. Effect of PDGF in combination with insulin (A), IGF-1 (B), or IGF-2 (C) on osteoblast survival *in vitro*.

Mouse osteoblasts were cultured as described in *Materials and Methods* in the presence of increasing concentrations of insulin, IGF-1, or IGF-2, alone or in combination with PDGF (10^{-9} M). Vehicle (CMRL-1066 medium) was used as a control. After 48 h, cell survival was assessed by the MTT cell survival assay. Each point is mean ± SEM of six wells. Each experiment was repeated three times.

TABLE 2-4. Effects of combinations of growth factors and cytokines on osteoblast survival

Treatment	% Survival after 48 h
IGF-I (10^{-9} M)	52 ± 3
IGF-I (10^{-9} M) + TGF-β (10^{-11} M)	51 ± 2
IGF-I (10^{-9} M) + EGF (10^{-9} M)	52 ± 4
IGF-I (10^{-9} M) + IL-1α (10^{-11})	54 ± 1
IGF-I (10^{-9} M) + IL-11 (10^{-9} M)	50 ± 5
IGF-I (10^{-9} M) + bFGF (20 ng/ml)	66 ± 2
IGF-I (10^{-9} M) + bFGF (20 ng/ml) + PDGF (10^{-9} M)	67 ± 1
IGF-II (10^{-7} M)	62 ± 4
IGF-II (10^{-7} M) + TGF-β (10^{-11} M)	60 ± 1
IGF-II (10^{-7} M) + EGF (10^{-9} M)	63 ± 5
IGF-II (10^{-7} M) + IL-1α (10^{-11})	61 ± 3
IGF-II (10^{-7} M) + IL-11 (10^{-9} M)	60 ± 2
IGF-II (10^{-7} M) + bFGF (20 ng/ml)	72 ± 2
IGF-II (10^{-7} M) + bFGF (20 ng/ml) + PDGF (10^{-9} M)	71 ± 3
Insulin (10^{-6} M)	55 ± 4
Insulin (10^{-6} M) + TGF-β (10^{-11} M)	54 ± 3
Insulin (10^{-6} M) + EGF (10^{-9} M)	52 ± 2
Insulin (10^{-6} M) + IL-1α (10^{-11})	55 ± 1
Insulin (10^{-6} M) + IL-11 (10^{-9} M)	56 ± 3
Insulin (10^{-6} M) + IGF-I	58 ± 4
Insulin (10^{-6} M) + IGF-II	57 ± 5
Insulin (10^{-6} M) + bFGF (20 ng/ml)	67 ± 7
Insulin (10^{-6} M) + bFGF (20 ng/ml) + PDGF (10^{-9} M)	65 ± 4

Mouse osteoblasts were cultured at a cell density of 10 000 cells per well of a 96 well plate in serum free CMRL-1066 medium in the presence of thymidine (10^{-3} M) and the above growth factors. After incubation for 48 h cell survival was assessed by MTT survival assay. The results are the mean ± SEM of 12 separate cultures.

2.3.6 Effect of αIR-3 on survival of mouse osteoblasts promoted by IGF-I and IGF-II

To determine which receptor was responsible for mediating the effects of the IGFs and insulin on osteoblast survival a murine monoclonal antibody, αIR-3 raised against the type I IGF receptor, was utilized. This antibody binds specifically to the type I receptor and does not cross-react with either the type II IGF or insulin receptor.

As shown in Fig. 2-10, αIR-3 (1.0 μg/ml) was a potent competitive inhibitor of both IGF-I and IGF-II mediated cell survival, indicating that in serum free culture both IGF-I and IGF-II mediate their effects on osteoblast survival by interacting with the type I IGF receptor. However αIR-3 did not completely inhibit the survival promoting effects of insulin, which suggests that insulin may be acting via an interaction with insulin receptors. αIR-3 had no effect on bFGF-mediated survival and only partially blocked the bFGF/IGF-I combination on osteoblast survival, which suggests that bFGF mediates its effects via an interaction with FGF receptors (Fig. 2-10). Finally, αIR3 blocked the action of PDGF/IGF-I on osteoblast survival and indicates that PDGF maybe altering the number and/or affinity of the type I IGF receptors on murine osteoblasts (Fig. 2-10).

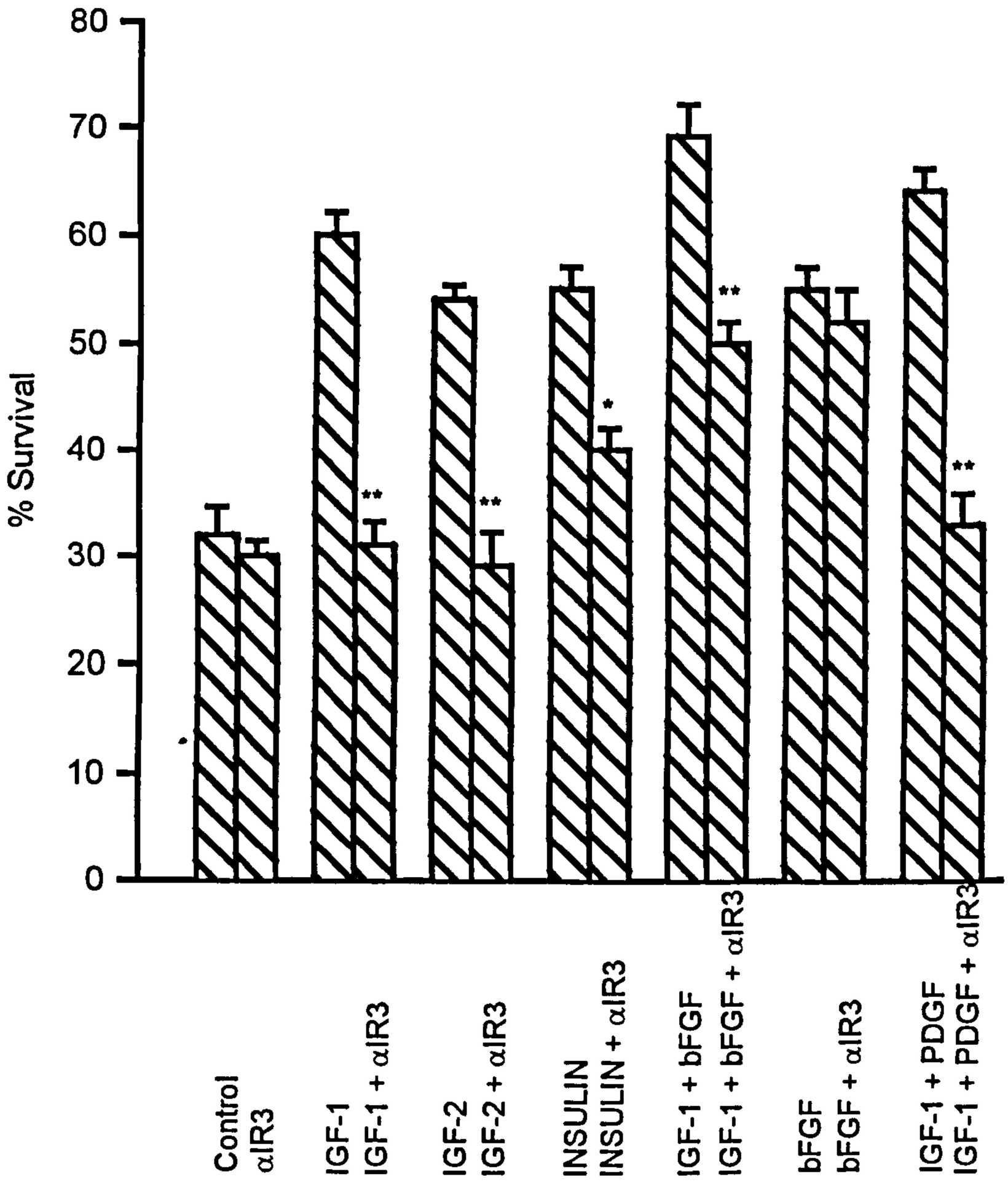


FIG. 2-10 Effects of αIR-3 on osteoblast survival.
Mouse osteoblasts were cultured at a cell density of 10000 cells/well in a 96-well plate in the presence of α-IR3 (1 μg/ml) and IGF-I (10^{-8} M), IGF-II (10^{-7} M), insulin (10^{-6} M), bFGF (20 ng/ml), bFGF/IGF-I, or PDGF (10^{-9} M)/IGF-I. Vehicle (CMRL-1066 medium) was used as a control. After 48 h in culture, cell survival was assessed by the MTT cell survival assay. Data are expressed as the mean ± SEM of six wells. *, $P<0.05$; **, $P<0.01$ (compared with treatment in the absence of αIR3).

2.4 DISCUSSION

This study shows that osteoblast survival *in vitro* is significantly increased by IGF-I, IGF-II, bFGF and insulin. Whilst PDGF was without effect on its own, this GF as well as bFGF enhanced the survival promoting effects of the IGFs and insulin although they were unable to achieve a 100% survival rate. It is increasingly believed that most normal cell deaths in invertebrate and vertebrate development depend on the activation of a suicide program in the cells that die (Wyllie and Kerr, 1980; Oppenheim *et al.*, 1990). The findings from these *in vitro* studies indicate that these GFs seem to promote survival by inhibiting programmed cell death rather than inducing proliferation as non-radioactive thymidine effectively prevented the proliferative effects of the GFs. A variety of other GFs, cytokines and osteotropic hormones had no effect on either osteoblast survival or apoptosis. The only factor that induced osteoblast apoptosis in this study was the immunoregulatory cytokine, TNF-α. In contrast to these results it has been demonstrated using TUNEL that both IL-6 and PTH suppress apoptosis in both mouse MC3T3-E1 and in human MG63 cells (Jilka *et al.*, 1998; 1999).

The effects of the IGFs on osteoblast survival is in agreement with their activity on the survival of oligodendrocytes (Barres *et al.*, 1992) and rat schwann cell precursors (Gavrilovic *et al.*, 1995). However, a notable difference between these reports and the present study was that the IGFs were capable of promoting a 100% survival rate of neuronal-derived cells as opposed to the 60-70% survival of osteoblasts achieved in the present study.

It would appear that insulin can promote survival of osteoblasts by binding to its own receptor; because insulin had a significant effect at a 10^{-8} M concentration, which is sufficient to bind to insulin receptors but not IGF-I receptors (Sara and Hall, 1990). This is supported by the fact that insulin receptors have been demonstrated on rodent osteoblasts and insulin can maintain the growth of these cells at a similar concentration (Hickman and McElduff, 1989). Although osteoblasts express type I and II IGF receptors (Slootweg *et al.*, 1990; Centrella *et al.*, 1990), the role of the type II receptor in mediating the metabolic and proliferative activities of IGF-II are controversial. It is known that the type I IGF receptor is recognized by both IGF-I and -II and insulin (Neely *et al.*, 1991) and several lines of evidence from this study indicate that the type I IGF receptor is responsible for mediating the effects of IGF-I and -II as well as high concentrations of insulin. Firstly the

order of potency in stimulating osteoblast survival (IGF-I> IGF-II>insulin) is similar to the relative affinities of these hormones for binding to the type I IGF receptor (Rechler *et al.*, 1980) and is consistent with a common mechanism involving this receptor. Similar potencies have been reported for the effects of the IGFs and insulin on other cellular activities and the type I IGF receptor seems to mediate the responses in these cell types (Jacobs *et al.*, 1986). Secondly, the findings that the combination of IGF-I and IGF-II did not enhance the level of osteoblast survival over that produced by IGF-I suggests that the type II receptor is not involved in the response. Thirdly, αIR-3, a monoclonal antibody specific for the type 1 IGF receptor, inhibited the survival promoting effects of both IGFs and almost prevented those of insulin. The finding in the present study that bFGF increased the survival of osteoblasts is similar to the situation for schwann cell precursors (Jessen *et al.*, 1994). Recent studies have demonstrated that the calcium binding protein, calbindin-D_{28k} is expressed by osteoblasts and suppresses apoptosis by inhibiting caspase-3 activity (Bellido *et al.*, 2000). Both IGF-I and FGF induce calbindin-D_{28k} expression and it has been suggested that calbindin-D_{28k} may mediate the antiapoptotic effects of IGF-I and FGF on osteoblasts (Bellido *et al.*, 2000).

Although PDGF has been shown to stimulate some cells to make IGF-I (Clemmons, 1985), this GF inhibits the synthesis of IGF-I and IGF-II by osteoblasts (Gabbitas *et al.*, 1994). This may explain why PDGF was incapable of inducing osteoblast survival on its own. The mechanism underlying the synergy between IGFs and FGFs/PDGF may be due in part to the ability of these GFs to modulate type-I IGF receptors. It has been shown for example, that bFGF and PDGF increase the number of type-I IGF receptors expressed by purified glial cells derived from hypothalamic cultures without affecting receptor affinity (Pons and Torres-Aleman, 1992). It is therefore conceivable that in the experiments reported here, bFGF/PDGF may increase type-I IGF receptor number or affinity, resulting in an enhanced response to IGF-I, IGF-II and insulin. This is supported by the fact that the type I receptor antibody, αIR3, prevented the survival enhancing effects of both bFGF/IGF-I and PDGF/IGF-I.

The action of IGFs is known to be regulated by the synthesis and secretion of one of six IGF-binding proteins (IGFBPs). Furthermore bFGF has been reported to modulate the synthesis of one of the IGFBPs in purified hypothalamic neural crest cell cultures. Modulation of these proteins by bFGF/PDGF therefore represents another way in which

these GFs might alter cellular responses to IGFs (Pons and Torres-Aleman, 1992; Ernst and Rodan, 1990).

In skeletal tissue osteoblasts express messenger RNA transcripts for IGF-I, IGF-II, bFGF and PDGF and the proteins have been shown to influence osteoblast proliferation and bone matrix synthesis (Centrella *et al.*, 1992; McCarthy *et al.*, 1989; Globus *et al.*, 1989). Furthermore studies on the quantification and characterization of GFs present in human bone has revealed that human bone matrix contains multiple GFs including IGF-I, IGF-II, TGF-β, bFGF and PDGF. IGF-II and TGF-β are the two most abundant GFs present in human bone while basic FGF, PDGF and IGF-I are several-fold less abundant (Mohan and Baylink, 1991).

Interestingly TGF-β was the only GF present in bone matrix which did not have any effect on either osteoblast survival or PCD in this study. This is in contrast to the results of Jilka *et al.* (1998) in which TGF-β promoted survival of the MC3T3-E1 preosteoblastic cell line. Osteoblast production of IGF-I and IGF-II is stimulated by TGF-β (Linkhart and Keffer, 1991) but the absence of a survival promoting effect suggests that this GF is unable to induce a single cell to produce enough IGF-I or IGF-II to save itself in microculture. The effects of TGF-β on apoptosis and cell survival are variable and seem to depend upon cell phenotype. For example, whilst it induces PCD in a variety of epithelial and myeloid cells (Bursch *et al.*, 1993; Taetle *et al.*, 1993), it prevents the process in synovial cells (Kawakami *et al.*, 1996), and has no effect on the survival of teratocarcinoma cells (Granerus *et al.*, 1995), which is similar to its effect on osteoblasts in this study . A possible explanation is unclear although it may relate to the density at which the cells are cultured as Mathieu *et al.*, (1995) have found that PCD *in vitro* exhibits a correlation with this parameter.

Since complete survival of osteoblasts in this study could only be achieved using culture medium supplemented with FCS, this would suggest either that unidentified GFs or extracellular matrix (ECM) components are responsible for promoting osteoblast survival. Among cells that have been shown to require survival factors, in no case is a single signalling molecule on its own capable of promoting long-term survival in culture (Dohrmann *et al.*, 1986; Sendnter *et al.*, 1991). The significance of the ECM in cell survival has recently been demonstrated for endothelial cells which rapidly undergo PCD in the absence of integrin mediated adhesion with components of the ECM (Meredith *et al.*,

1993). More recently, fibronectin has been shown to be a survival factor for mature differentiated rat osteoblasts (Globus *et al.*, 1998). It seems possible that all cells require multiple survival factors for long-term survival.

The effects of inflammatory cytokine TNF-α on osteoblast apoptosis in this study is in accordance with previous studies demonstrating its apoptotic effects in the MC3T3-E1 cell line (Jilka *et al.*, 1998). Apoptosis in response to TNF-α has been demonstrated in several other mammalian cell lines, including the human leukemia cell lines HL-60 and U937 (Elias *et al.*, 1988) and the murine fibrosarcoma cell lines L929 and WEHI (Fehsel *et al.*, 1991).

Whilst biochemical and morphological features of osteoblast apoptosis were detected, DNA fragmentation was not detected by agarose gel electrophoresis in cultures stimulated with TNF-α. It may be that DNA fragmentation is more apparent in cells which have detached from the tissue culture dish, which were not collected in this study or that osteoblasts need to be cultured at a lower cell density. DNA fragmentation, however, seems not to be an invariable feature of PCD (Lockshin *et al.*, 1991) and condensation of the chromatin at the membrane of an apoptotic nucleus is not always associated with activation of an endonuclease with subsequent DNA degradation (Oberhammer *et al.*, 1994). TNF-α inhibits bone formation and has been found to inhibit collagen synthesis and alkaline phosphatase activity in osteoblasts, actions which contrast with those GFs which promote osteoblast survival in this study. This suggests that those factors exerting a catabolic action on osteoblasts may also induce PCD whilst conversely agents with an anabolic action may promote survival.

3. Autocrine Signals Promote Osteoblast Survival in Culture.

3.1 INTRODUCTION

Many studies on the control of cell survival have demonstrated that certain mammalian cell types have specific survival requirements and require signals from other cell types in order to survive, just as cells require signals for cell growth (Baserga, 1985). Oligodendrocytes and their precursors require specific growth factors such as IGF-I, IGF-II and PDGF (Barres *et al.*, 1992). Colony stimulating factors promote cell survival of hematopoietic cells by suppressing apoptosis (Williams *et al.*, 1990) and M-CSF promotes osteoclast survival (Fuller *et al.*, 1993). Similarly, developing neurons require neurotrophic factors (Levi-Montalcini, 1987). If deprived of their specific survival factors these cells seem to die by PCD. In addition other factors such as plasma proteinase inhibitors may be important determinants of cell survival (Ikari *et al.*, 2001).

On the basis of such studies it has been proposed that most mammalian cells are programmed to undergo PCD in the absence of survival signals from other cells (Raff, 1992). In the case of chondrocytes and lens epithelial cells it has been demonstrated that these cells can survive in the absence of other cell types, serum and exogenous proteins if cultured at high density, but they undergo PCD if cultured in these conditions at low cell density (Ishizaki *et al.*, 1993; Ishizaki *et al.*, 1994). It would appear that chondrocytes are sensitive to growth factor deprivation and toxic oxygen metabolites derived from molecular oxygen, i.e., hydroxyl radicals or hydrogen peroxide although their relative contribution to chondrocyte survival remains uncertain. Cartilage is an unusual tissue in that it contains only a single cell type and is not innervated, vascularized or penetrated by lymphatic vessels (Goss, 1978; Fawcett, 1986). It is possible that chondrocytes have adapted to their environment in some manner which has permitted them to survive under these conditions. *over

In addition to the growth factors discussed in chapter 2 other factors may have effects on osteoblast survival. Since bone contains different cell types, survival signals may act in a paracrine fashion to effect osteoblast survival. In this study the survival requirements of primary osteoblasts are assessed at different cell densities in the absence of

*

Actively metabolizing cells produce quantities of reactive oxygen species that lead to oxidative damage to proteins and macromolecules (Cadenas, 1989). Cells have evolved complex antioxidant defenses to protect themselves against the harmful effects of reactive oxygen species. Such antioxidants include the sulfhydryl containing amino acid cysteine (Cadenas, 1989) that combines with reactive oxygen to form cysteine sulfoxide. Cysteine has been shown to have an anti-apoptotic effect in cultures of resting chondrocytes which are sensitive to toxic oxygen metabolites (Tschan *et al.*, 1990). Intracellularly cysteine is utilized for the synthesis of cellular antioxidants such as glutathione. Thus addition of cysteine to cell cultures enables it to be utilized for the maintenance of cellular antioxidant potential, through direct inactivation of reactive oxygen and as an intermediate in the synthesis of glutathione.

growth factors and cytokines used in chapter 2. The aim was to establish whether osteoblast derived survival signals can act in an autocrine fashion to promote the survival of other osteoblasts, in a similar manner to chondrocytes and determine the survival requirements of these cells in culture.

3.2 Materials and Methods

3.2.1 Materials.

Terminal deoxynucleotidyl transferase, biotinylated dUTP, and streptavidin fluorescein were purchased from Boehringer Mannheim GmbH (U.K.). MTT and all cell culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The following anti-human polyclonal antibodies were purchased from R and D Systems, (Oxon,U.K): IGF-I, IGF-II, basic FGF, and PDGF. [^{3}H]-cytidine was purchased from Amersham Int. (U.K.)

3.2.2 Methods

3.2.2.1 Preparation of osteoblasts from neonatal mouse calvaria

Calvarial osteoblasts were prepared and characterized as described in section **2.2.2.1**. Osteoblasts were cultured in α-MEM containing 10% FBS and antibiotics for 4 days prior to use.

3.2.2.2 Preparation of osteoblast conditioned medium

Osteoblasts were cultured in 96-well tissue culture plates at 100 000 cells/well and medium was removed and replaced by fresh CMRL medium every 2 days. The conditioned medium was immediately transferred to low density cultures, which were fed with conditioned medium every two days.

3.2.2.3 Fractionation of Conditioned Medium by Ultrafiltration

1.5 ml of conditioned medium from high density osteoblast cultures was poured into the filter cup of a Millipore ultrafiltration unit (molecular mass cutoff 5 kDa), and centrifuged at 3,000 g for 45 min at room temperature. The concentrate (1.0 ml) and filtrate (0.5 ml) were sterilized by passage through a 0.22 μm millipore filter and immediately transferred to low density osteoblast cultures.

3.2.2.4 Osteoblast survival assays

For survival assays, osteoblasts were plated in 100 µl of CMRL-1066 medium with 1% FCS for 2 h to permit adhesion of osteoblasts. Thereafter media was replaced with serum free CMRL-1066 medium containing thymidine (10^{-3} M) to block cell proliferation, with or without cysteine or osteoblast conditioned medium (see below).

The human osteoblastic cell line MG63 was used in some experiments. These cells have a well characterized osteoblast phenotype and are frequently used to study the effects of cytokines and growth factors *in vitro*.

MTT assay. Cell survival was assayed in flat-bottomed 96 well microtitre plates. Cell survival was assessed by the MTT assay as described in section **2.2.2.2**.

3.2.2.5 Osteoblast Proliferation assay

Primary osteoblasts were plated at a density of 10000 cells/well of a 96 well plate and cultured for 48 h in CMRL-1066 medium with and without either 10% FCS or conditioned media from high density osteoblast cultures in the presence or absence of non-radioactive thymidine (10^{-3} M). Assessment of proliferation in the presence of non-radioactive thymidine was performed as described in section **2.2.2.3**.

3.2.2.6 Identification of apoptotic osteoblasts

(a) TUNEL assay: Apoptosis-induced DNA strand breaks were enzymatically labelled by a fluorescein isothiocyanate (FITC) *in situ* cell-death detection kit (Boehringer Mannheim, U.K.) based on the TUNEL technique which stains individual apoptotic nuclei with green fluorescence (Gavrieli *et al.*, 1992). Cells were fixed in 4% paraformaldehyde for 10 min, washed in 10 mM Tris-HCl, pH 8.0, and then permeabilized in 0.1% Triton X-100 in 0.1M sodium citrate for 5 min on ice. After washing in 10 mM Tris-HCl, pH 8.0 the cells were incubated 60 min with TdT and FITC-dUTP at 37°C. After washing in PBS, the cells were examined using a Leica fluorescence microscope.

(b) DNA fragmentation: DNA fragmentation was analyzed by agarose gel electrophoresis as described in section **2.2.2.4**. Primary mouse osteoblasts were cultured in serum free CMRL-1066 medium for 1 or 2 days and DNA extracted from the combined adherent and non adherent cell fractions.

3.2.2.7 Statistical analysis

Data are expressed as the means ± SEM of 6 cultures/group. Each experiment was repeated three times. Differences between control and treatment groups were determined by the Mann-Whitney U test.

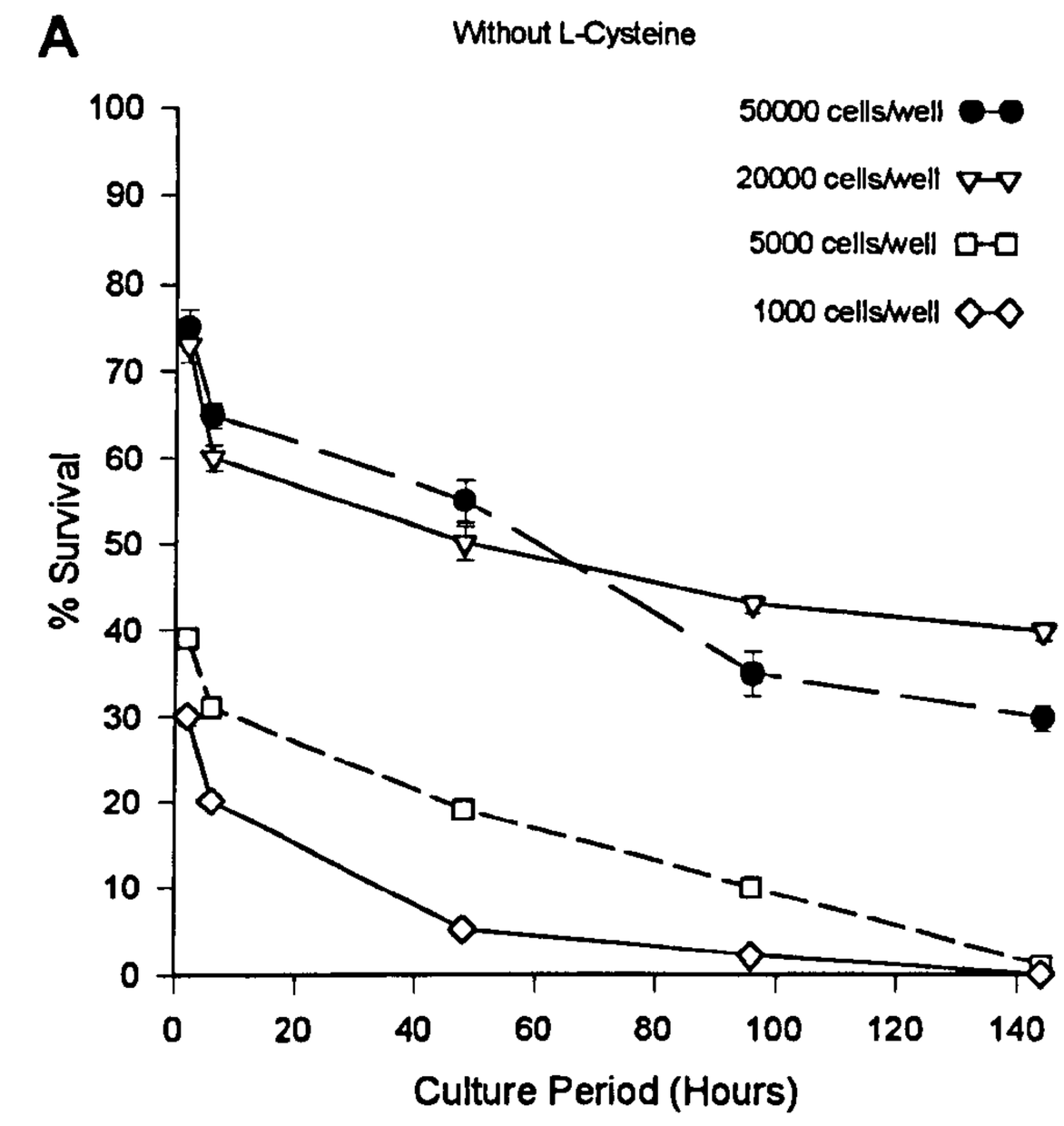
3.3 RESULTS

3.3.1 Characterization of murine osteoblasts

Unstimulated cultures of primary mouse osteoblasts were characterized by histochemical staining for alkaline phosphatase and intracellular accumulation of cAMP in response to PTH. 94.2 ± 2.4% of cells from six separate bone cell preparations were found to be strongly positive for alkaline phosphatase. Treatment of primary mouse osteoblast cultures with PTH (10^{-8} M) for 10 min induced a cAMP level of 10.7 ± 1.8 pmol/ml compared with a control level of less than 0.34 pmol/ml.

3.3.2 Osteoblast survival in Protein-free medium is cell-density dependent

To study the survival requirements of osteoblasts in culture, osteoblasts prepared from calvaria of 2-day-old mice were cultured at various cell densities in serum-free CMRL medium containing thymidine (10^{-3} M) but no added protein in 96-well plates. Cell survival was assessed by the MTT assay after various times. Viable cells converted the MTT into a dark blue reaction product, while dead cells remained uncoloured. The survival of osteoblasts was cell-density dependent: when plated at $\geq 2 \times 10^4$ cells/well, 30% of the cells survived for upto 144 h, but when plated at $\leq 5 \times 10^3$ cells/well, all the cells had died by 144 h (Fig 3-1a). To confirm the hypothesis that the dead osteoblasts were undergoing apoptosis rather than necrosis, osteoblasts were examined by TUNEL labelling of nuclei with FITC-dUTP. A large proportion of the cells had clear-cut staining indicative of chromatin condensation at the nuclear membrane (Fig. 3-2). The occurrence of apoptosis, as opposed to necrosis, in primary mouse osteoblasts was confirmed by the demonstration of 180 bp DNA fragments, and multimers thereof, in cell extracts (Fig. 3-3).



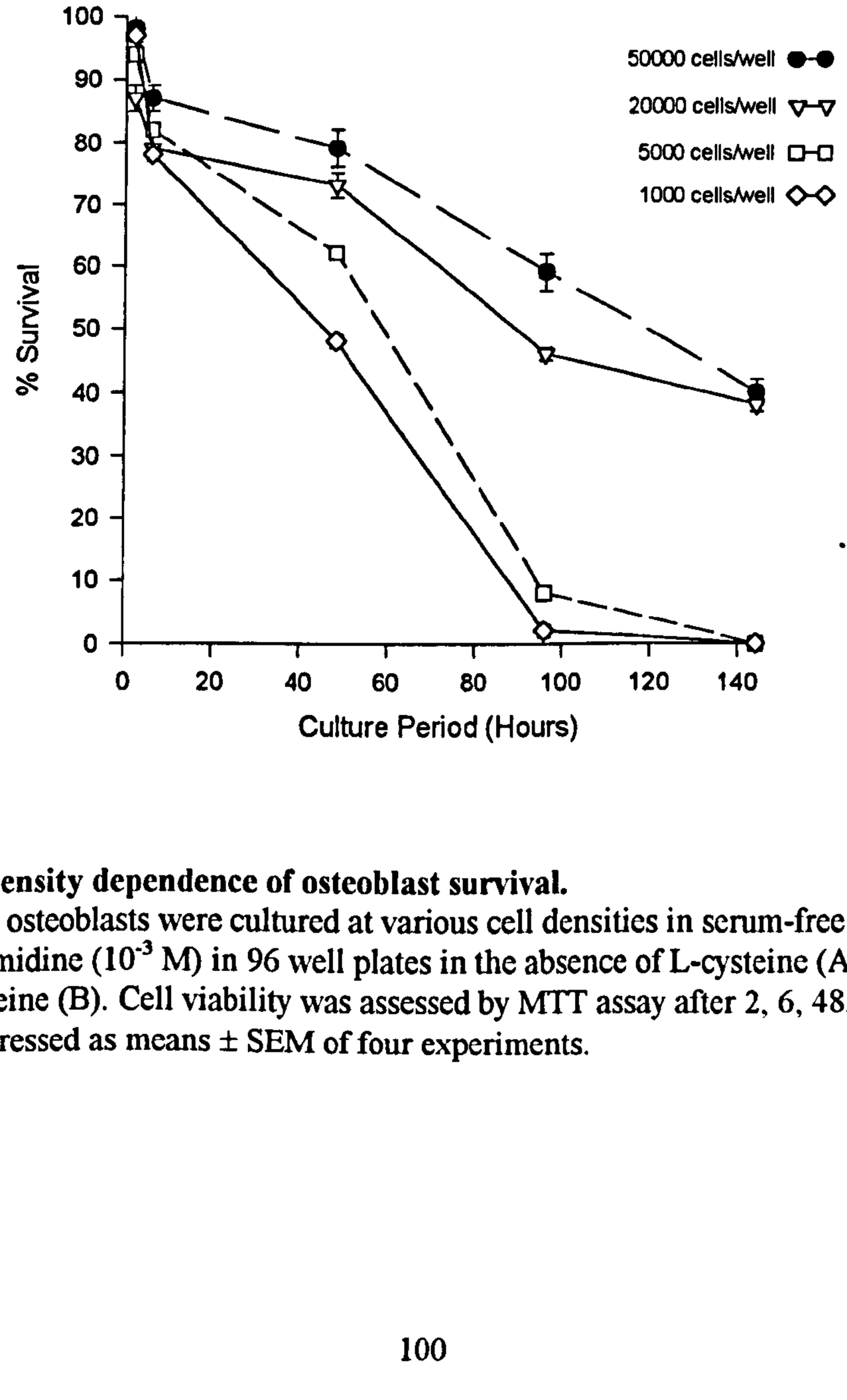


Fig. 3-1 Cell-density dependence of osteoblast survival.
Primary mouse osteoblasts were cultured at various cell densities in serum-free CMRL-1066 medium containing thymidine (10^{-3} M) in 96 well plates in the absence of L-cysteine (A) and in the presence of 1mM L-cysteine (B). Cell viability was assessed by MTT assay after 2, 6, 48, 96 and 144 hours. Results are expressed as means ± SEM of four experiments.

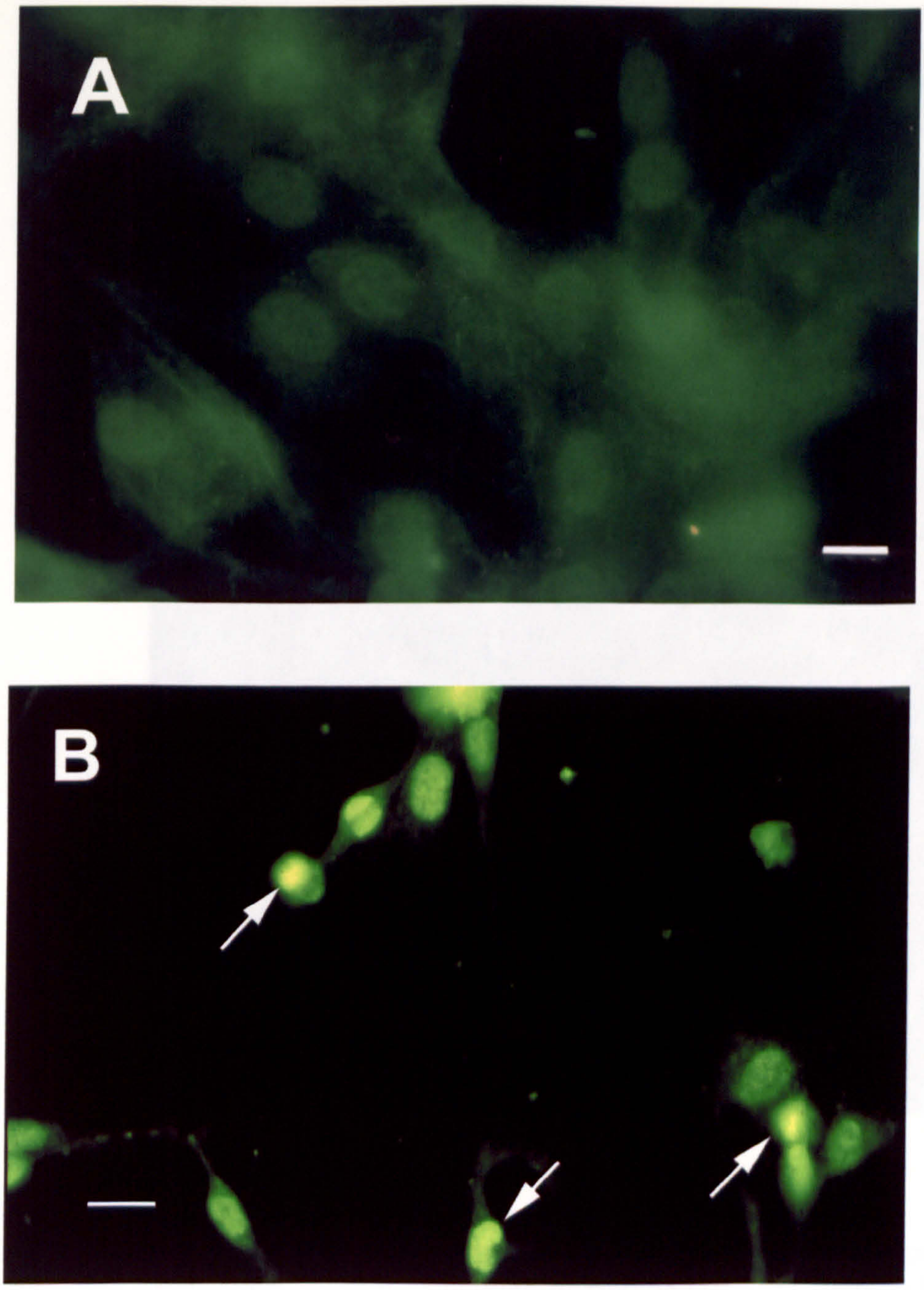


Fig. 3-2. Detection of apoptotic osteoblasts by TUNEL.
Mouse osteoblasts were cultured in CMRL-1066 medium containing thymidine (10^{-3} M) and 1 mM cysteine at high density (5 x 10^4 cells/well) or low density (5 x 10^3 cells/well). After 4 days, the cells were fixed with 4% paraformaldehyde, permeabilized by incubation with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, and incubated for 30 min with FITC-labelled d-UTP. A, Typical example of osteoblasts showing negligible DNA fragmentation; B, cells show intense fluoresence of condensed nuclear chromatin (arrows), indicative of cells in the later stages of apoptosis. Bar = 10 μm.

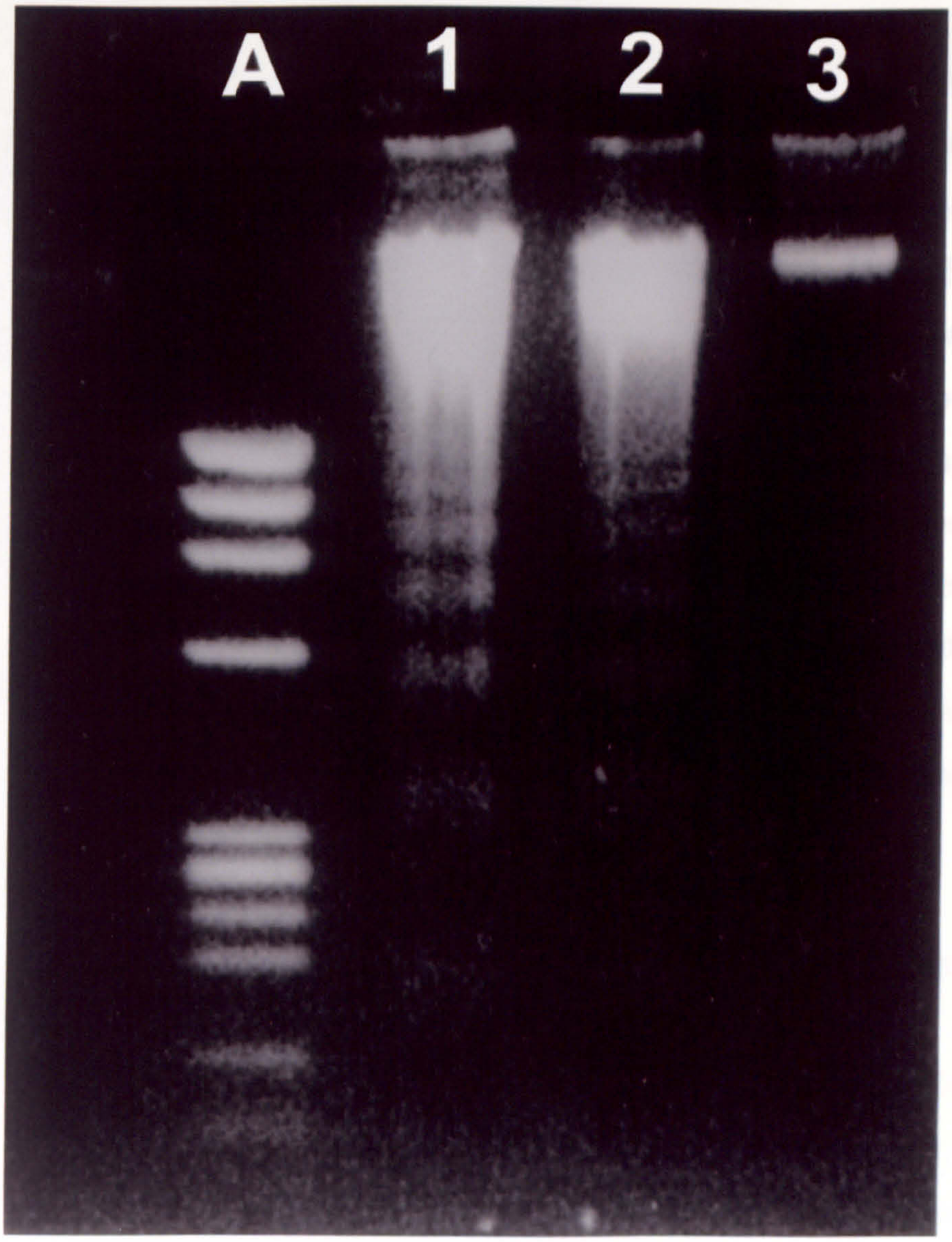


Fig. 3-3. Apoptosis of osteoblasts detected by DNA fragmentation.
Primary mouse osteoblasts were cultured at a density of 5000 cells per well of 96 well plates in CMRL-1066 medium containing thymidine (10^{-3} M), with and without CM from high density osteoblast cultures for 1 or 2 days. DNA was extracted and 5 μg was analyzed by electophoresis on a 2% agarose gel. DNA was visualized with ethidium bromide (0.5 μg/ml). Lanes 1 and 2, DNA isolated from osteoblasts cultured in the absence of CM from high density cultures after 1 and 2 days respectively. Lanes 3, DNA isolated from osteoblasts after 2 days in culture with CM from high density osteoblast cultures. Lane A, DNA size marker (∅X174 Hae III digest).

3.3.3 Antioxidants prevent osteoblast cell death

As many inhibitors of apoptosis have antioxidant properties the effects of a variety of antioxidants on osteoblast survival were assessed. The addition of cysteine to the culture medium greatly enhanced the survival of mouse osteoblasts, especially when cultured at $\leq 5 \times 10^3$ cells/well, but only for the first 48 h of culture (Fig. 3-1b). Thereafter, most of the cells died with the characteristic features of apoptosis. Cystine also promoted survival, but to a lesser extent than cysteine (Table 3-1). The ability of cysteine to promote survival apparently depended on the intracellular conversion by glutathione synthase to the antioxidant glutathione, as an inhibitor of glutathione synthase, BSO, prevented this effect (Table 3-1). Glutathione in reduced form (GSH) was as effective as cysteine, while its oxidized form (GSSG) was less effective; neither of their effects were affected by BSO (Table 3-1). When superoxide dismutase was added to culture media, cell viability was not improved whereas catalase clearly protected the osteoblasts. Superoxide dismutase also had no effect in conjunction with catalase (Table 3-1).

3.3.4 Osteoblasts support one another's survival in culture by secreting survival factors that are not antioxidants.

The finding that osteoblasts survived longer when cultured at high density suggests that the cells in high density cultures promote one another's survival through cell-cell contact survival factors or by secreting survival-promoting factors. Consistent with the latter suggestion, culture medium from high density (10^5 cells/well) osteoblast cultures promoted the survival of low density osteoblast cultures. When osteoblasts were cultured at a cell density of 5×10^4 cells/well in the presence of conditioned medium 63 ± 4% of osteoblasts survived after 144 h in culture (Fig. 3-4) compared with a control level of 30 ± 3% in the absence of conditioned medium (Fig. 3-1A). Addition of cysteine to the conditioned medium from high density cultures did not further enhance survival of mouse osteoblasts cultured at 5×10^4 cells/well. Addition of cysteine to the conditioned medium was requred to save mouse osteoblasts cultured at $\leq 2\times10^4$ cells/well (Fig. 3-4).

When conditioned medium from high density cultures of either mouse or human osteoblasts was fractionated by ultrafiltration using a membrane with a molecular mass cut-off of 5 kDa, the survival promoting activity was recovered in the concentrate and not in the filtrate. When cultured at a cell density of 5×10^3 in the presence of concentrate 83 ± 5% of

osteoblasts survived after 48 h and 47 ± 4% after 144 h compared with a control level of 48 ± 3% for osteoblasts cultured after 48 h and 8 ± 1% after 144 h in the absence of conditioned medium (Fig. 3-5). However, when osteoblasts were cultured in the presence of the filtrate only 56 ± 3% survived after 48 h and 13 ± 2% survival was obsurved after 144 h. This suggested that one or more osteoblast derived growth factors was responsible for promoting osteoblast survival.

TABLE 3-1 Effects of antioxidants on the survival of osteoblasts cultured at low density.

Treatment	Conc. (M)	% Survival
Control		30 ± 1.6
BSO	$2x10^{-4}$	30 ± 2.2
Cysteine	10^{-3}	63 ± 1.5 **
Cystine	10^{-3}	52 ± 2.1**
Cysteine + BSO	$10^{-3} + 2x10^{-4}$	34 ± 2.3
Cystine + BSO	$10^{-3} + 2x10^{-4}$	27 ± 2.6
GSH	10^{-3}	60 ± 2.4**
GSH + BSO	$10^{-3} + 2x10^{-4}$	57 ± 1.7**
GSSG	10^{-3}	40 ± 1.1*
GSSG + BSO	$10^{-3} + 2x10^{-4}$	38 ± 1.0*
SOD	50 U	32 ± 2.0
Catalase	50 U	56 ± 2.7**
SOD + Catalase	50 U + 50 U	58 ± 2.1**

Mouse osteoblasts were cultured for 48 h in CMRL 1066 medium at $5x10^3$ cells per well, and their viability was assessed by MTT assay. BSO, buthionine-sulphoximine; GSH, reduced glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase. The results are the means ± SEM of quadruplicate cultures *P<0.05, **P<0.01 significantly different from controls.

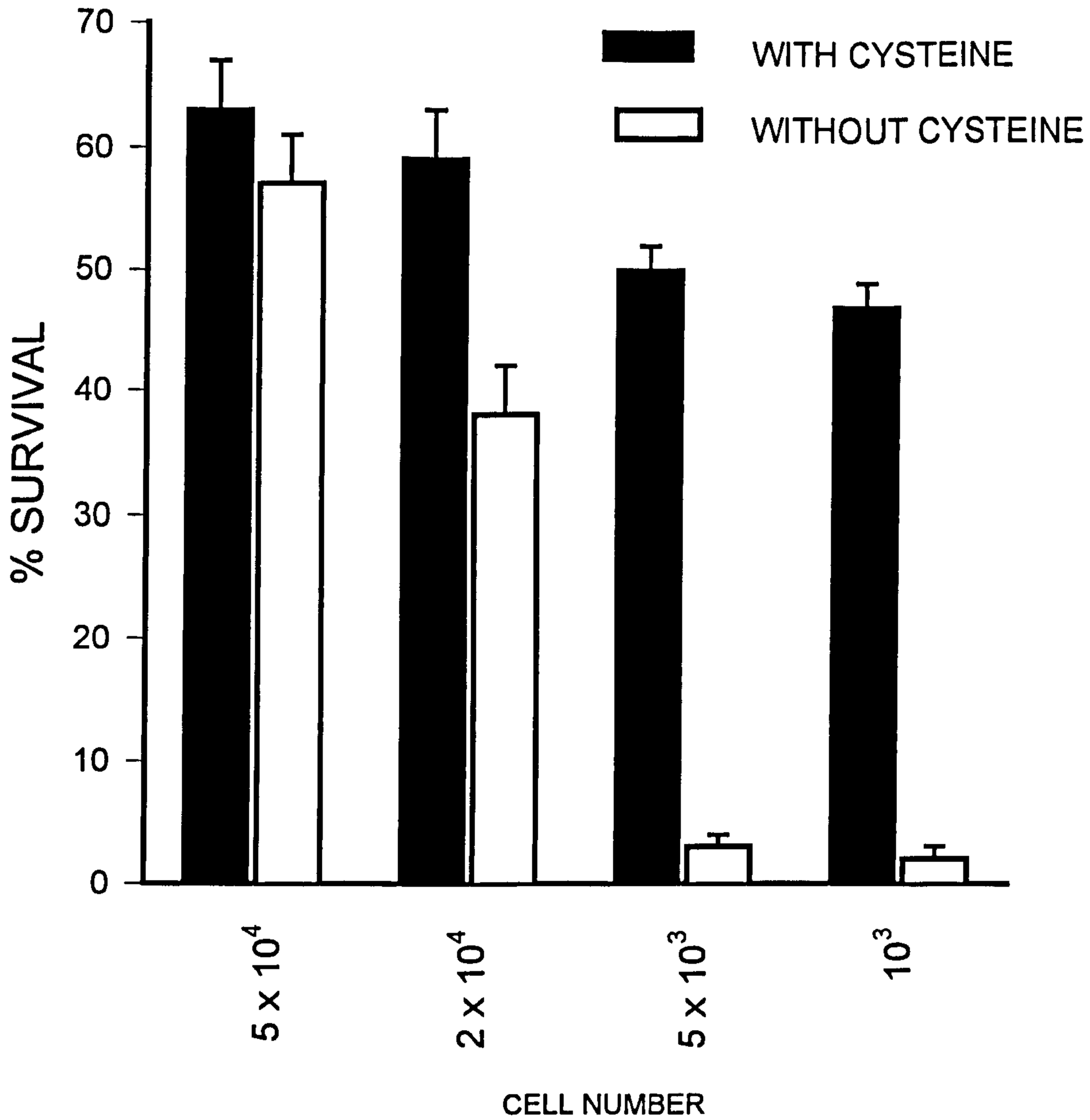


Fig. 3-4. Effects of osteoblast conditioned medium on osteoblast survival.
Neonatal mouse osteoblasts were cultured at various cell densities in 96-well plates with or without 1 mM cysteine in the presence of conditioned medium from high density (10^5 cells/well) osteoblast cultures. MTT assays were performed after 144 hrs.

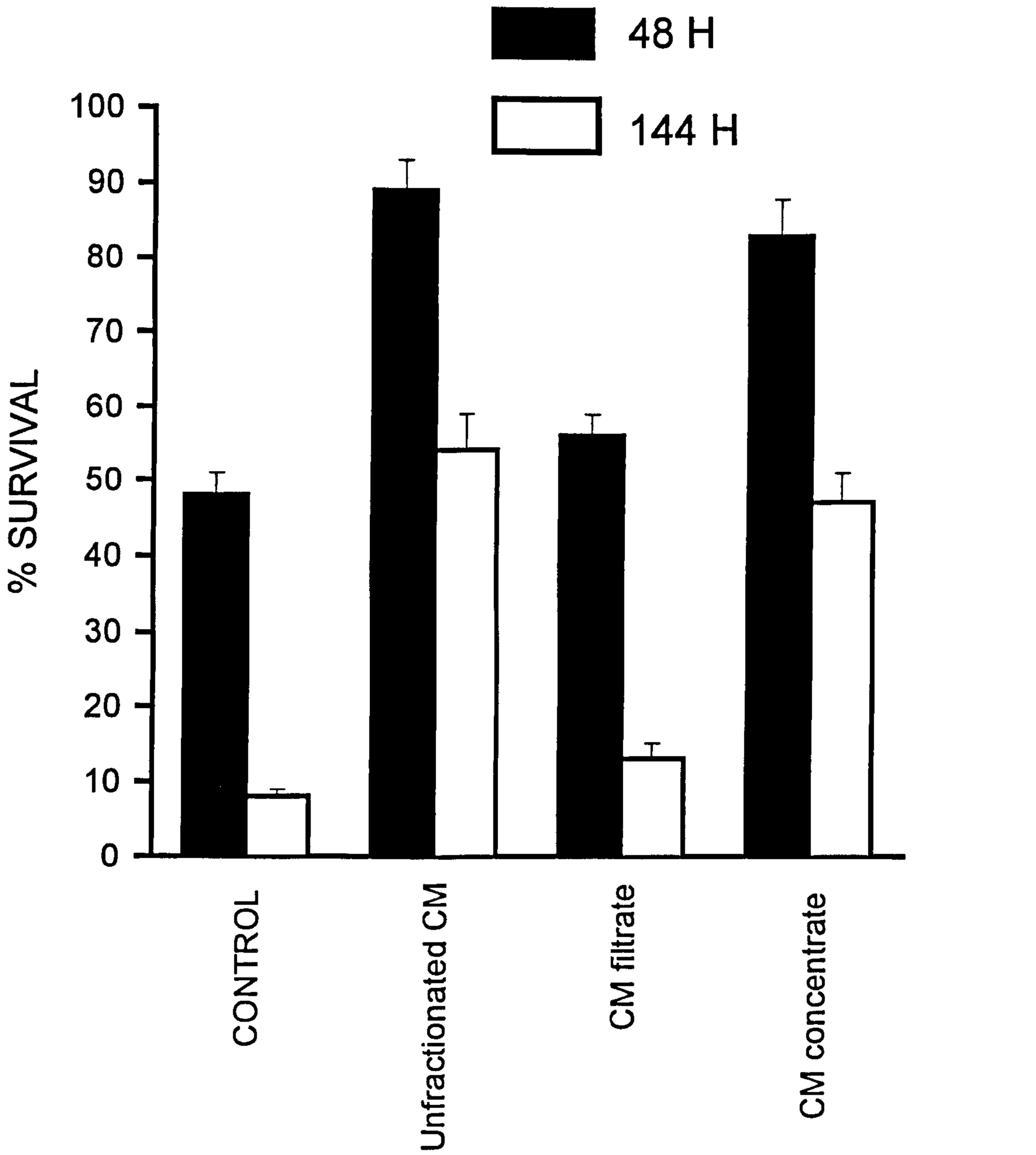


Fig. 3-5. Ultrafiltration of conditioned medium.
Conditioned medium (CM) was collected from high density cultures (10^5 cells/well) mouse osteoblast cultures and fractionated by ultrafiltration through a membrane that retains molecules above 5 kDa molecular weight. The concentrate and the filtrate were immediately tested for their ability to promote the survival of mouse osteoblasts in low density (5 x 10^3 cells/well) cultures in the presence of 1 mM cysteine. MTT assays were performed after 48 and 144 hrs.

Labelling osteoblast DNA with [^{3}H]-cytidine confirmed that non-radioactive thymidine effectively blocked the effects of FCS and conditioned medium from high density cultures on osteoblast proliferation (Table 3-2).

TABLE 3-2 Effects of non-radioactive thymidine (10^{-3} M) on osteoblast proliferation in the presence/absence of either 2% FCS or conditioned medium from high density osteoblast cultures.

Treatment	Non-radioactive thymidine (+/-)	dpm
Control	+	2056 ± 234
Control	-	1975 ± 216
2% FCS	+	2213 ± 222
2% FCS	-	14324 ± 654
CM	+	2351 ± 176
CM	-	9865 ± 543

Primary mouse osteoblasts were cultured at a density of 10,000 cells/well of a 96-well plate in CMRL medium with and without non-radioactive thymidine for 48 h. Cells were labelled by addition of [^{3}H]-cytidine. CM: conditioned medium from high density osteoblast cultures. The results are the mean ± SEM of quadruplicate cultures.

3.3.5 Effects of neutralizing antibodies to growth factors on osteoblast survival

To determine which particular growth factors might be responsible for promoting osteoblast survival the effects of neutralizing antibodies to those growth factors that are produced by osteoblasts were assessed. Antibodies to IGFs-I and -II were capable of preventing the survival promoting effects of conditioned medium on low density osteoblast cultures whilst antibodies to PDGF and basic FGF were without effect and did not augment the inhibitory activity of the IGF antibodies. (Table 3-3). When the various antibodies were added to high density MG63 osteoblast cultures in the absence of conditioned medium, a similar effect was observed (Table 3-3).

Table 3-3 Effects of neutralizing antibodies on the survival promoting effects of osteoblast conditioned medium on osteoblasts.

	% Survival of Osteoblasts		
Antibody Type	Low Density Cultures	Low Density cultures + Conditioned medium	High Density MG63 Cultures
Control	8 ± 3	51 ± 6	44 ± 5
IGF-I	12 ± 4	12 ± 4**	14 ± 2**
IGF-II	9 ± 3	14 ± 3**	9 ± 2**
PDGF	14 ± 4	52 ± 6	46 ± 5
bFGF	7 ± 3	54 ± 5	51 ± 4

Murine osteoblasts were cultured in 96 well plates at low cell density (5 x 10^3 cells/well) and MG63 osteoblasts were cultured at high cell density (5 x 10^4 cells/density) for 2 days. Conditioned medium from high density MG-63 cultures was added to the low density cultures as indicated. Human polyclonal antibodies to bFGF, IGFs -I and -II were added at a final concentration of 2 μg/ml and PDGF at 5 μg/ml. The results are the means ± SEM of quadruplicate cultures **, $P < 0.01$ significantly different from the control.

3.4 DISCUSSION

These studies demonstrate that osteoblasts do not require signals from other cell types to survive if cultured at high cell density in the absence of serum, exogenous proteins or non-protein molecules. This is similar to survival characteristics of both chondrocytes and lens epithelial cells (Tschan *et al.*, 1990; Ishizaki *et al.*, 1993). The studies also show that osteoblasts require assistance from other osteoblasts to survive in culture: the cells die rapidly when cultured at low cell density, but can be saved by conditioned medium from high density osteoblast cultures. The apparent rapid cell death prominent in cells cultured at a density on ≤ 5x10^3 cells per well within the first three hours of switching to serum free medium may be due to a lack of adhesion resulting from a lack of cell-cell contact survival

factors. The deaths involve activation of an endogenous endonuclease as shown by DNA laddering, a characteristic feature of apoptosis (Wyllie *et al.*, 1980b) suggesting that the cells die by active PCD.

The survival of a given cell may be determined by its balance of reactive oxygen intermediates (ROI) and antioxidants. The demonstration that a variety of antioxidants that scavenge or detoxify ROIs suggests that the latter may be responsible for mediating osteoblast death in the low density cultures.

Since sulfhydryl reagents can act as oxygen radical scavengers (Cadenas, 1989) these observations suggested such radicals might be a possible cause of the observed cytotoxicity. Oxygen radicals arise from molecular oxygen by one-electron transfer reactions. For example, $O_2^-\bullet$ and $OH\bullet$ can be formed in the presence of Fe^{2+} according to:

$$O_2 + Fe^{2+} \rightarrow O_2^-\bullet + Fe^{3+} \qquad (1)$$

$$2O_2^-\bullet + 2H+ \rightarrow O_2 + H_2O_2 \qquad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow OH\bullet + OH^- + Fe^{3+} \qquad (3)$$

Toxic oxygen-derived compounds can be eliminated enzymatically. $O_2^-\bullet$ is decomposed by superoxide dismutase, and catalase degrades H_2O_2 to oxygen and water, thus also preventing the production of $OH\bullet$ (formula 3). Both of these enzymes are known to protect cells from oxygen toxicity (Cadenas, 1989). These results show that oxygen toxicity caused the loss of viable cells in serum-free osteoblast culture. The findings also emphasize the importance of H_2O_2 or $OH\bullet$, rather than $O_2^-\bullet$.

Although the results demonstrate that antioxidants are important for the survival of osteoblasts in low density cultures ($\leq 5 \times 10^3$ cells/well), they are not enough: extracellular signalling molecules are also required. This is supported by the findings that (a) the survival-promoting activity in the conditioned medium from high density osteoblast cultures collaborates with antioxidants such as cysteine and is associated with molecules that are larger than 5 kDa, and (b), this activity is prevented by antibodies to known growth factors. While conditioned medium from high density osteoblast cultures does not require antioxidants to promote the survival of either mouse or human osteoblasts cultured at 5×10^4 cells/well, it does require antioxidants to promote the survival of osteoblasts cultured at

$\leq 5 \times 10^3$ cells/well. It can be concluded that osteoblasts, like chondrocytes and many other cells require signals from their neighbours in order to avoid PCD, at least in culture. Furthermore these signals do not function solely as antioxidants, and osteoblasts themselves can secrete such signals. However, the evidence from this study showing that conditioned medium from high density osteoblast cultures was unable to achieve a 100% osteoblast survival rate, suggests that other factors, possibly cell-to-cell contact factors play a role in promoting the survival of osteoblasts cultured at high cell densities.

Of the neutralizing antibodies to growth factors that were tested, both the IGF-I and -II antibodies prevented the survival promoting activity of osteoblast conditioned medium. Osteoblasts have been shown to make both these GFs (McCarthy *et al.*, 1989) and it seems likely that these GFs promote osteoblast survival *in vivo*. Whilst these GFs stimulate osteoblast proliferation (McCarthy *et al.*, 1989; Ernst and Rodan, 1990), their effects on survival and proliferation were distinguished by incorporating non-radioactive thymidine into the culture medium which effectively blocked the proliferative effects of the conditioned medium. Although bFGF, TGF-β, PDGF, IGF-I and IGF-II are all present in the bloodstream, it is apparent that the paracrine biosynthesis of GFs is more important in the modulation of cellular activity (Holly and Wass, 1989). It seems likely that GFs released from the extracellular matrix and neighbouring cells are responsible for promoting the survival of osteoblasts in bone as they do *in vitro*. The IGFs have previously been shown to be important survival factors during normal tissue development of other cell types such as Schwann cells (Delaney *et al.*, 1999) and also in the prevention of cytokine-mediated cell death in pancreatic islets (Hill *et al.*, 1999). The IGFs have also been shown to prevent apoptosis in many tumour cell lines. It has been demonstrated that IGF-I is the most important growth factor present in serum that prevents apoptosis in neuroblastoma cells (Van Golen and Feldman, 2000) and this is associated with regulation of the levels of the antiapoptotic proteins Bcl-2 and Bcl-X_L. IGF-I also protects colon cancer cells from death factor-induced apoptosis (Remacle-Bonnet *et al.*, 2000). In contrast to the survival promoting effects of IGF-I found in this study, it has been demonstrated that stimulation of human osteoblasts with IGF-I induces expression of Fas and subsequently increased their susceptibility to Fas-mediated apoptosis when cocultured with $FasL^+$ cells (Kawakami *et al.*, 1998). It remains to be seen whether IGF-I increases susceptibility to Fas-mediated apoptosis in murine osteoblasts.

The isolation procedure used in this study will yield osteoblasts at different stages of differentiation from osteoprogenitor through to mature osteoblasts. A significant proportion of these cells stain strongly for alkaline phosphatase suggesting they are of the preosteoblast/osteoblast stage of differentiation. Although it is not certain whether osteoprogenitors express IGF-I or IGF-I receptors evidence suggests that genes for regulatory proteins such as FGF receptor-I are modulated during progression from late osteoprogenitor to preosteoblast and osteoblast stages (Aubin, 1998). Therefore in the culture system used in this study these stages may be responsive to the survival promoting effects of IGF-I.

Although no inhibitory activity was found with either bFGF or PDGF neutralizing antibodies this does not exclude a role for the involvement of these GFs *in vivo*. There are an increasing number of examples where several distinct extracelluar signalling molecules have been shown to collaborate to promote cell survival in culture (Barres *et al.*, 1993), presumably reflecting the advantages of combinatorial control.

Although an assessment was not made as to whether other cells within the bone microenvironment can promote the survival of osteoblasts, it has previously been shown that lens epithelial cells can promote chondrocyte survival and vice versa (Ishizaki *et al.*, 1993). One theoretical advantage of having cells depend on signals from their neighbours for survival is that any cell that ends up in an abnormal location would fail to receive the survival signals it requires and would consequently die; because cells seem to require a combination of signals for sustained survival, at least in culture, a relatively small selection of signalling molecules used in different combinations could specifically control the survival of many distinct cell types.

Interestingly, osteoblast apoptosis has been shown to occur *in vivo* with a frequency of ~ 0.6% (Jilka *et al.*, 1998) and it has been concluded that only about 15% of terminally differentiated osteoblasts survive to become osteocytes *in vivo* (Parfitt, 1990), although what activates the death program in these cells remains unknown. An explanation may involve the loss of expression of type I IGF receptors by terminally differentiated osteoblasts similar to the loss of expression of bFGF receptors by hypertrophic chondrocytes (Iwamoto, 1991).

Osteoblast apoptosis has also been shown to be important in the pathogenesis of certain metabolic bone diseases. Studies have shown that exposure to high doses of

glucocorticoids such as dexamethasone increase apoptosis of mature osteoblasts and osteocytes resulting in glucocorticoid-induced osteoporosis (Weinstein *et al.*, 1998). Interestingly, it has been suggested that glucocoticoids antagonize the effects of IGF-I (Delaney and Canalis, 1995). Osteoblast apoptosis may also be important in postmenopausal osteoporosis since it has been shown that estrogen inhibits osteocyte and osteoblast apoptosis (Manolagas *et al.*, 1999).

4. The Resorptive Actions of Interleukin-11 On Bone *in vitro*

4.1 INTRODUCTION

Bone formation and resorption are coupled through the actions of several locally produced growth factors and cytokines. Unlike other cytokines involved in hematopoiesis, IL-11 is not produced by monocytes or lymphocytes, but its expression is restricted to certain cells of the mesenchymal lineage (Maier *et al.*, 1993). There is evidence to suggest that IL-11, like IL-6, is an important osteotropic factor. IL-11 receptor transcripts are present in chondroblastic and osteoblastic progenitor cells during mouse embryogenesis (Elias *et al.*, 1995). IL-11 is produced by both primary osteoblasts and human osteosarcoma SaOS-2 cells in response to bone resorbing agents (Elias *et al.*, 1995; Romas *et al.*, 1996) and Girasole *et al.* (1994) demonstrated that IL-11 dose-dependently stimulates osteoclast-like multinucleated cell (OCL) formation in co-cultures of mouse osteoblasts and bone marrow cells. They also reported that a monoclonal anti-IL-11 antibody inhibited OCL formation induced by several osteotropic factors.

Bone resorption involves a series of events, the central step being the removal of bone matrix by osteoclasts. Osteoblasts play an accessory role in bone resorption by releasing matrix metalloproteinases (MMPs) that degrade the surface osteoid layer (principally type-I collagen), facilitating the access of osteoclasts to the mineralized bone (Chambers *et al.*, 1985). Bone resorption is also governed by the recruitment of new osteoclasts from progenitor cells of the mononuclear phagocyte system (Suda *et al.*, 1992). The mononuclear progenitors are disseminated via the bloodstream and deposited in the mesenchyme surrounding the bone rudiments where they proliferate and differentiate into (pre)osteoclasts prior to migrating to future resorption sites (Blavier and Delaisse 1995). MMPs have been shown to play an important role in 1,25-$(OH)_2D_3$-mediated migration and fusion of osteoclast precursors (Blavier and Delaisse 1995).

It is known that prostaglandins (PGs), the cyclo-oxygenase (CO) products of arachidonic acid metabolism, are powerful mediators of bone resorption and that a variety of agents that stimulate resorption do so by generating PGs in the microenvironment of

bone-resorbing cells. More recently products of the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism, namely 5-hydroxyeicosatetraenoic acid (5-HETE) and the peptido-leukotrienes (LTs) LTB_4, LTC_4, LTD_4, and LTE_4 have been found to enhance bone resorption but their contibution to cytokine-induced resorption has not been established (Garcia *et al.*, 1996). Although IL-11 has been shown to stimulate osteoclast formation (Girasole *et al.*, 1994), it is not known if the activity of IL-11 is restricted to this stage of bone resorption or whether MMPs and products of arachidonic acid metabolism play a role in mediating the resorptive activity of IL-11.

The aims of this study were to use a variety of discriminatory assays to better define the mechanisms of action of IL-11 in bone resorption.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Human recombinant IL-11 and IL-1α were gifts from the Genetics Institute (Cambridge, MA) and Dr. J. Saklatvala, Strangeways Research Laboratory (Cambridge, UK) respectively. The specific MMP inhibitor, CT1166 was a gift from Dr. A. Docherty, Cell Tech, UK. CT1166, a concentration-dependent selective inhibitor of gelatinases A and B, inhibits MMP activity at a 10^{-7} M concentration (Hill *et al.*, 1995). Modified Biggers (BGJ) medium was obtained from Flow Laboratories (Irvine, UK). $^{45}CaCl_2$ and ^{14}C were purchased from Amersham International, Aylesbury, United Kingdom. Indomethacin and α-MEM were obtained from Sigma Chemical Company (Poole, Dorset, UK). The 5-LO inhibitors, BWA70C and MK886 were gifts from Professor B. Henderson, Eastman Dental Institute (UK). BWA70C is a selective inhibitor of the enzyme 5-LO whilst MK886 is a selective inhibitor of the 5-LO activating protein (FLAP). The later protein binds to 5-LO and is an obligatory participant in the activity of this enzyme. Inhibition of the interaction between FLAP and 5-LO blocks the synthesis of lipoxygenase products. Mice (CD-1 strain) were purchased from Charles River Breeding Laboratories (U.K.).

4.2.2 Methods

4.2.2.1 Neonatal calvarial assay

Bone resorption was assessed by analysing $^{45}Ca^{2+}$ release from cultured neonatal mouse calvarial bones (Reynolds and Dingle, 1970). Briefly, 1-day-old mice were injected subcutaneously with 0.1 megabecquerels $^{45}CaCl_2$ or 1 megabecquerel $^{45}CaCl_2$ for the kinetic studies. After 6 days, the mice were sacrificed and the calvariae were excised and dissected into two equal halves and precultured in modified BGJ medium (2 ml) containing 26 mM $NaHCO_3$, 0.85 mM ascorbic acid, 1.4 mM L-glutamine, 5% FBS (Globepharm, Surrey, United Kingdom), and indomethacin (1 μM) for 24 h. Bones were subsequently cultured in pairs in fresh modified BGJ medium (2 ml) for up to 8 days with a media change every 2 days. Mobilization of radioactivity was expressed as the percent release of initial isotope (calculated as the sum of radioactivity in medium and bone after culture). To determine $^{45}Ca^{2+}$ release due to passive exchange of isotope, two bones from each litter were devitalized by three cycles of freeze-thawing. The percent release from the devitalized bone was subtracted from each living bone to give the amount of cell-mediated resorption.

4.2.2.2 Preparation of osteoblasts from neonatal mouse calvariae

Calvarial osteoblasts were prepared and characterized as described in section **2.2.2.1.** Cells released by the last two collagenase digestions were washed and grown in DMEM containing 10% FBS and antibiotics for 2 days before use. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2-95% air.

4.2.2.3 Preparation of collagen films

Radiolabelled collagen films were prepared as described previously (Gavrilovic *et al*, 1985). Aliquots of ^{14}C-acetylated collagen (rat skin type I; 150 μg in 300 μl of 10 mM phosphate buffer, pH 7.4, containing 300 mM NaCl and 0.02% sodium azide) were dispensed into tissue culture wells (Linbro, 16 mm diam) and dried at 37°C.

4.2.2.4 Preparation of acid-treated serum

To destroy serum inhibitors of neutral proteinases, aliquots (20 ml) of heat-inactivated rabbit serum (Globepharm) were acidified to pH 3.2 with 1 M HCl and incubated for 35 min at 37°C. The pH was then returned to 7.4 with 1 M NaOH.

4.2.2.5 Culture of osteoblasts on collagen films

Osteoblasts (1 x 10^5/well) were settled onto collagen films in 1 ml of DMEM plus 10% (v/v) FCS, incubated for 16 h at 37°C and washed with serum-free DMEM. Cells were then cultured in DMEM (1 ml) supplemented with 5% (v/v) acid-treated rabbit serum as described above. Either 1,25-$(OH)_2D_3$ or IL-11 alone (final concentration 10^{-8} M and 10^{-9} M respectively, added in 5 µl of ethanol) or either compound in the presence of the respective MMP or arachidonic acid inhibitors were then added to the wells and the cultures maintained at 37°C for 48 h. At the end of the culture period the media were centrifuged (15 min, 1200x*g*) to remove any collagen fibrils and radioactivity released during collagen degradation quantified by liquid scintillation counting. Residual collagen was digested with bacterial collagenase (50 µg/ml) and assayed for radioactivity. Collagenolysis was expressed as radioactivity released from the films as a percentage of the total ± SEM.

4.2.2.6 Isolated osteoclast assay

The osteoclast bone resorption assay is based on the ability of isolated osteoclasts to resorb devitalized cortical bone, dentine, or ivory slices *in vitro* (Boyde *et al.*, 1984). Ivory slices, 200 µm thick, were cut with a low speed water-cooled diamond saw (Isomet, Buehler, Coventry, United Kingdom) from a 1 cm^2 rod. Ivory slices were chosen because they are free of vascular systems and pre-existing resorbing surfaces. Osteoclasts were prepared from either 2- to 3-day-old mice. After killing the animals, femurs and tibias were removed, and osteoclasts were isolated by curetting the bones into 4 ml PBS and agitating the cell suspension with a pipette. Larger fragments were allowed to settle for 10 sec before 500 µl aliquots of the supernatant cell suspension were immediately transferred to 6 wells of 24 well culture dishes (Costar, Cambridge, MA), each containing a single ivory slice. Cells were allowed to settle and attach for 25 min at 37°C. The substrate was then washed free of nonadherent cells, and then the various test substances were added to the cultures which

were subsequently incubated for 48 h in a humidified atmosphere of 5% CO_2-95% air at 37°C in 500 µl α-MEM supplemented with 5% FBS, 2.0 g/L $NaHCO_3$, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Due to the variability in the number of osteoclasts isolated from each mouse, a single experiment consisted of 6 ivory slices bearing the cells from 1 mouse, with 3 slices for each control and test variable. Each experimental variable was repeated 4 times.

At the completion of the 48 h culture period, the cells were removed from the ivory slices, which were then stained with toluidine blue to count the resorption lacunae. The method used for the precise quantitation of the resorptive capacity involved estimating the surface area of the resorption lacunae by image analysis (TC Image, Foster Finlay Associates, United Kingdom). To test the indirect responsiveness of osteoclasts to IL-11, osteoclasts obtained after a short sedimentation time (25 min) were co-cultured with neonatal mouse calvarial osteoblasts (1 x 10^5 cells/well) for 48 h. Bone resorption was quantified as above.

4.2.2.7 Murine bone marrow cell cultures

A mouse bone marrow culture system was used to assess osteoclast differentiation (Takahashi *et al.*, 1988). 5- to 6-week-old mice were killed by cervical dislocation and the femurs and tibias were removed and dissected free of adherent tissue. The epiphyses were removed and the marrow cavity was flushed with 1 ml α-MEM using a sterile 30-gauge needle. The bone marrow suspension was aspirated up and down with a 1 ml pipette and the resulting single cell suspension washed 3 times by centrifugation at 300xg. The marrow cells were plated in 24-well dishes, with each well containing either a Thermanox coverslip (Nunc, Naperville, IL) or a 1 cm^2 ivory slice, at a density of 2 x 10^6 cells/well in 0.5 ml α-MEM containing 10% FBS and 10^{-6} M dexamethasone. The cultures were incubated in the presence of IL-11 and/or 1,25-$(OH)_2D_3$ (10^{-8}). Cultures were fed every 3 days by replacing 250 µl culture medium with fresh medium and hormone or vehicle. After 8 days, the cultures on the coverslips were stained for TRAP and the number of TRAP-positive multinucleated cells (MNCs; three or more nuclei) was counted. The cells were removed from the ivory slices after 8 days, and the substrate was stained with toluidine blue and

examined for the presence of resorption lacunae by light microscopy. The surface area of bone resorption was quantified as before.

4.2.2.8 Fetal metatarsal long bone assay

The 3 middle metatarsals of each hindlimb of 17-day-old mouse embryos (day of vaginal plug discovery equals day 0 of gestation) were dissected as a triad (Blavier and Delaisse, 1995). One triad of each pair was cultured as a control, the other as a test. The long bones were cultured without removal of the cartilaginous epiphyses, and care was taken not to damage the periosteum-perichondrium. Each triad was cultured in 1 ml CMRL 1066 medium supplemented with glutamine (200 mg/l), 10% heat inactivated FCS with and without either 1,25-$(OH)_2D_3$ (10^{-8} M) or IL-11 (10^{-9} M). Media were renewed every day. The three metatarsals were placed on a piece of lens paper, itself deposited upon a stainless steel grid, which was in turn suspended above the center well of the organ culture dish. The culture period was 3 days.

4.2.2.9 Preparation of tissue sections

Plastic sections for a detailed characterization of the migration pathway of the osteoclasts were prepared as described (Blavier and Delaisse, 1995). Metatarsals were fixed in 4% neutral buffered formalin for 18 h at 4°C, and embedded in glycolmethacrylate according to the instructions of the "Historesin Embedding Kit" (Reichert-Jung). Sections of 3 µm were cut with glass knives in a Reichert-Jung microtome (20/50, supercut), stained for TRAP and counterstained with haematoxylin (Van de Wijngaert and Burger, 1986).

4.2.2.10 Histomorphometry

The number of TRAP-positive cells and their nuclei were determined in 10 evenly spaced longitudinal sections per long bone rudiment. According to their location they were scored as (a) lying in the developing marrow cavity, that is the area of resorbing calcified cartilage surrounded by the thin bone collar; or (b) in the periosteum-perichondrium, that is, the soft tissue around the bone rudiment. The few cells lying within the (thin) bone collar were equally divided over the two compartments.

4.2.2.11 Statistical analysis

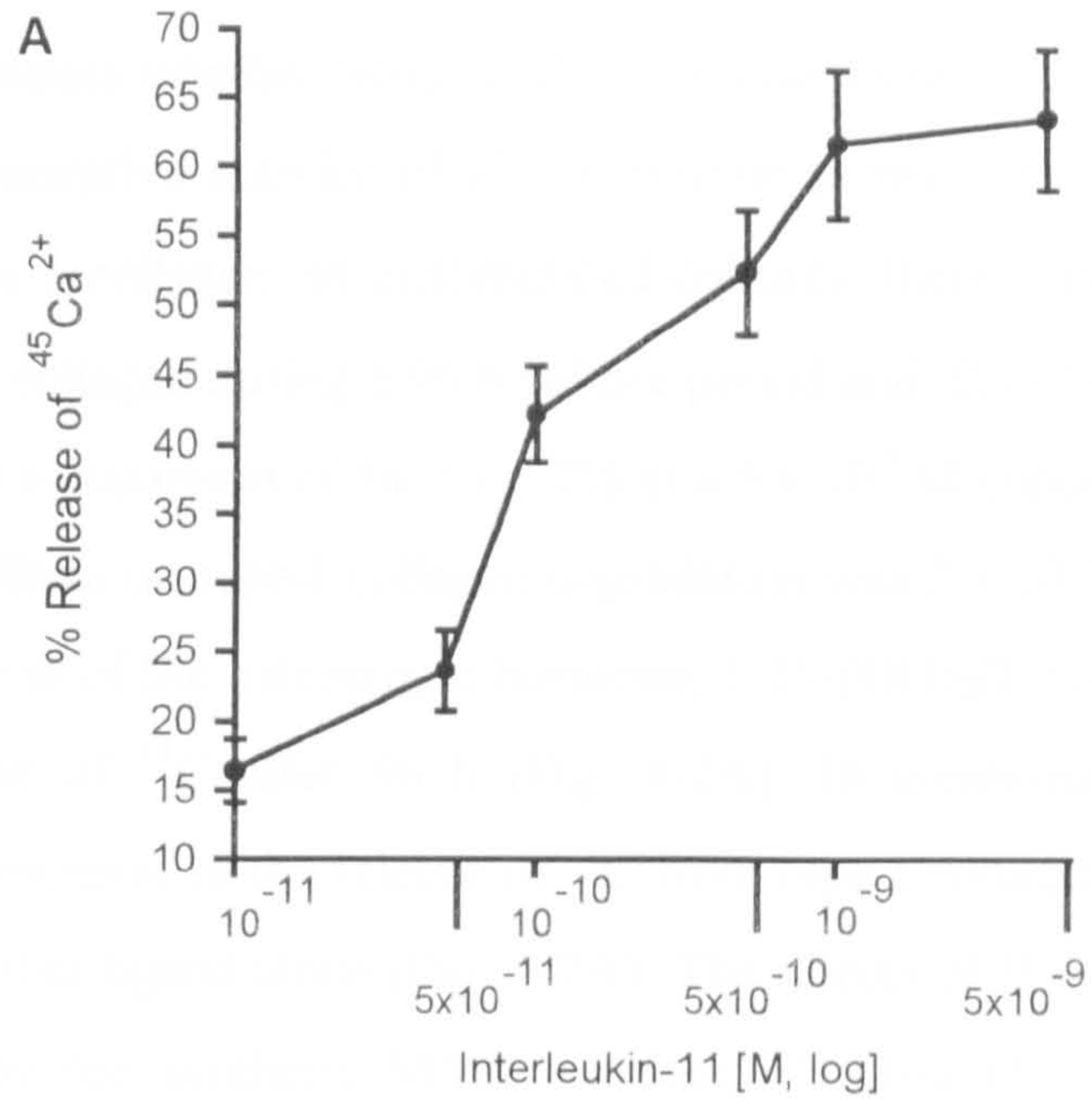
Differences between the control and treatment groups were determined by the Mann-Whitney U test.

4.3 RESULTS

4.3.1 Effects of IL-11 on calvarial bone resorption

The neonatal mouse calvarial assay was used to screen for activity modulating osteoclast differentiation and function. During an 8-day culture period, IL-11 dose-dependently increased the release of $^{45}Ca^{2+}$ from calvarial explants with a maximal effect of 62.5 ± 6.7 % at 5×10^{-9} M (Fig 4-1A.). The EC_{50} for IL-11 mediated $^{45}Ca^{2+}$ release was 10^{-10} M. As shown in Figure 4-1B the kinetics of $^{45}Ca^{2+}$ release from calvarial explants as a function of time (2, 4, 6, 8 days) demonstrated that the effects of IL-11 were different from those of three known resorptive agents namely PTH, 1,25-$(OH)_2D_3$ and IL-1α. Whilst the latter three agents stimulated $^{45}Ca^{2+}$ release throughout the culture period, IL-11 (10^{-9} M) had no effect on $^{45}Ca^{2+}$ release during the first 2 days of culture, but had a significant stimulatory effect during the subsequent culture period (Fig. 4-1B).

The specific MMP inhibitor, CT1166 (10^{-7} M) produced a 95.6 ± 6.7% inhibition in IL-11 (10^{-9} M)-stimulated $^{45}Ca^{2+}$ release over an 8 day culture period, whilst the PG synthesis inhibitor, indomethacin (10^{-6} M) produced a 92.7 ± 7.8% inhibition and the LT synthesis inhibitor, BWA70C (10^{-6} M) blocked the effects of IL-11 on $^{45}Ca^{2+}$ release by 33.8 ± 4.5%.



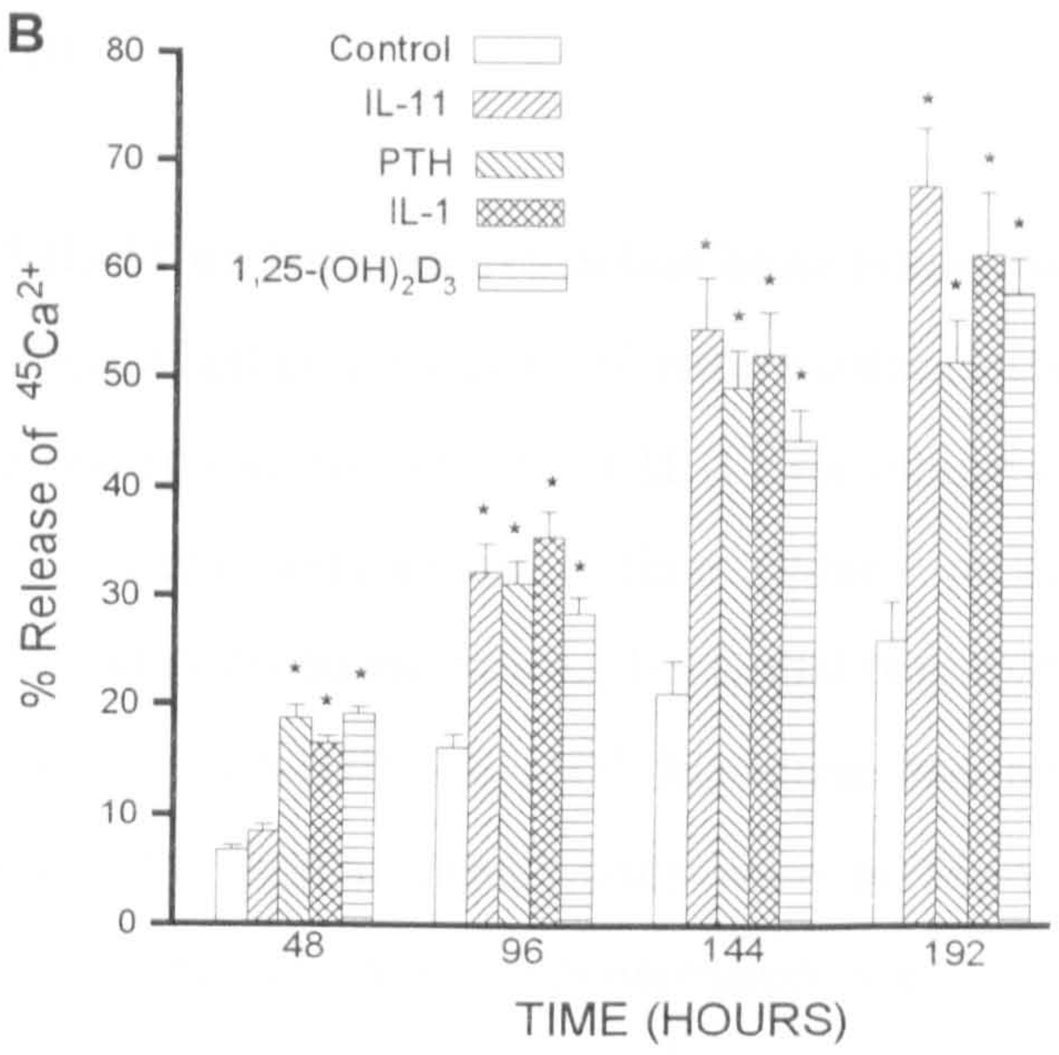


FIG. 4-1 Effects of IL-11 on $^{45}Ca^{2+}$ release from neonatal calvariae.

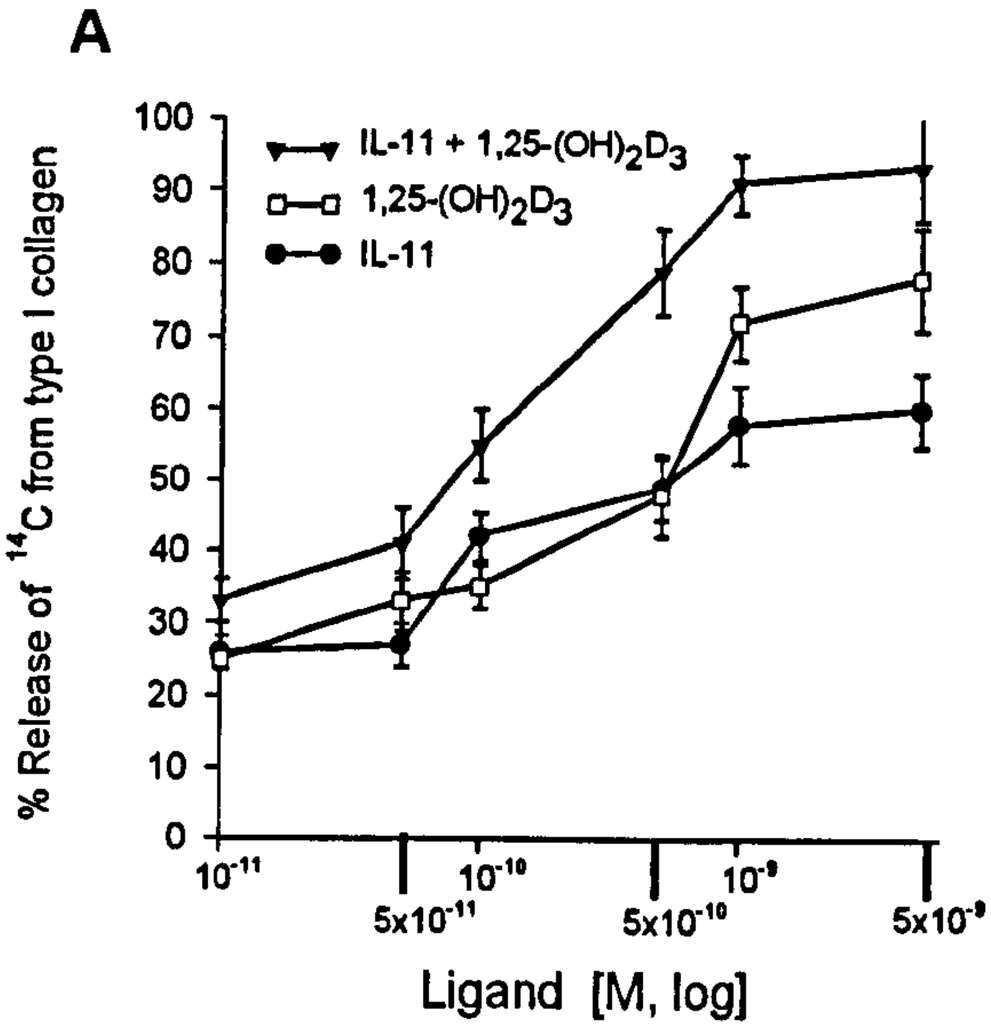
A $^{45}Ca^{2+}$ prelabelled mouse calvariae were cultured as described in *Materials and Methods*, with increasing concentrations of IL-11 for 8 days. Values are expressed as the mean percentage (± SEM) of radioisotope released from five pairs of cultured bones for each ligand concentration. The control release of $^{45}Ca^{2+}$ was 16.7 ± 2.3%. **B,** A comparison of the effects of IL-11, PTH, 1,25-$(OH)_2D_3$ and IL-1α on $^{45}Ca^{2+}$ release from neonatal mouse calvariae. $^{45}Ca^{2+}$ prelabelled mouse calvariae were cultured in the presence or absence of IL-11 (10^{-9} M), PTH (10^{-9} M), 1,25-$(OH)_2D_3$ (10^{-8} M) or IL-1α (10^{-10} M) for either 2,4,6 or 8 days. Values are expressed as the mean percentage (± SEM) of radioisotope released from five pairs of cultured bones for each time interval. The effect of IL-11 was not significant until 96 h.

4.3.2 Effects of IL-11 on the degradation of type-I collagen by mouse osteoblasts

To assess whether removal of the unmineralized osteoid layer of bone plays a part in the bone resorptive activity of IL-11, murine primary osteoblasts were cultured on ^{14}C-labelled type I collagen. In unstimulated cultures, there was a 24.1 ± 4.9% release of ^{14}C from type-I collagen during a 96 h culture period and IL-11 dose-dependently increased the release with a maximum of 58.0 ± 6.2% at a 5 x 10^{-9} M concentration (Fig. 4-2A). The EC_{50} for IL-11 effects on type-I collagen degradation was 2 x 10^{-10} M. The effects of IL-11 were less than those of the osteotropic hormone, 1,25-$(OH)_2D_3$ (10^{-8} M) which induced a 76.5 ± 7.1% release of ^{14}C after 96 h (Fig. 4-2A). In combination, IL-11 and 1,25-$(OH)_2D_3$ induced an increase in the release of ^{14}C from type I collagen films (91.7 ± 9.4%) over that seen with either ligand alone (Fig. 4-2A). The effects of IL-11 (10^{-9} M) on ^{14}C release were abrogated by the synthetic MMP inhibitor, CT1166 (10^{-7} M), whilst the PG inhibitor, indomethacin (10^{-6} M) and the LT synthesis inhibitor, BWA70C (10^{-6} M) were without effect (Fig. 4-2 B).

4.3.3 Effects of IL-11 on isolated osteoclast bone resorption

To ascertain whether activation of mature osteoclasts was responsible for the IL-11 stimulated bone resorption, the effects of IL-11 on mouse osteoclast populations obtained after a short (25 min) sedimentation time (which minimizes contamination by non-osteoclastic cells and consequent indirect hormonal responses) were examined. Neither IL-11 (5 x 10^{-9} M) nor 1,25-$(OH)_2D_3$ (10^{-8} M) alone had any effect on osteoclast lacunar resorption (Table 4-1). Similar experiments were performed in which mouse osteoclasts obtained after a short (20 min) sedimentation were incubated with primary mouse osteoblastic cells. Whilst 1,25-$(OH)_2D_3$ (10^{-8} M) stimulated osteoclast lacunar resorption in these co-culture experiments, IL-11 did not increase or decrease osteoclast lacunar resorption, its effects being similar to the unstimulated cultures (Table 4-1).



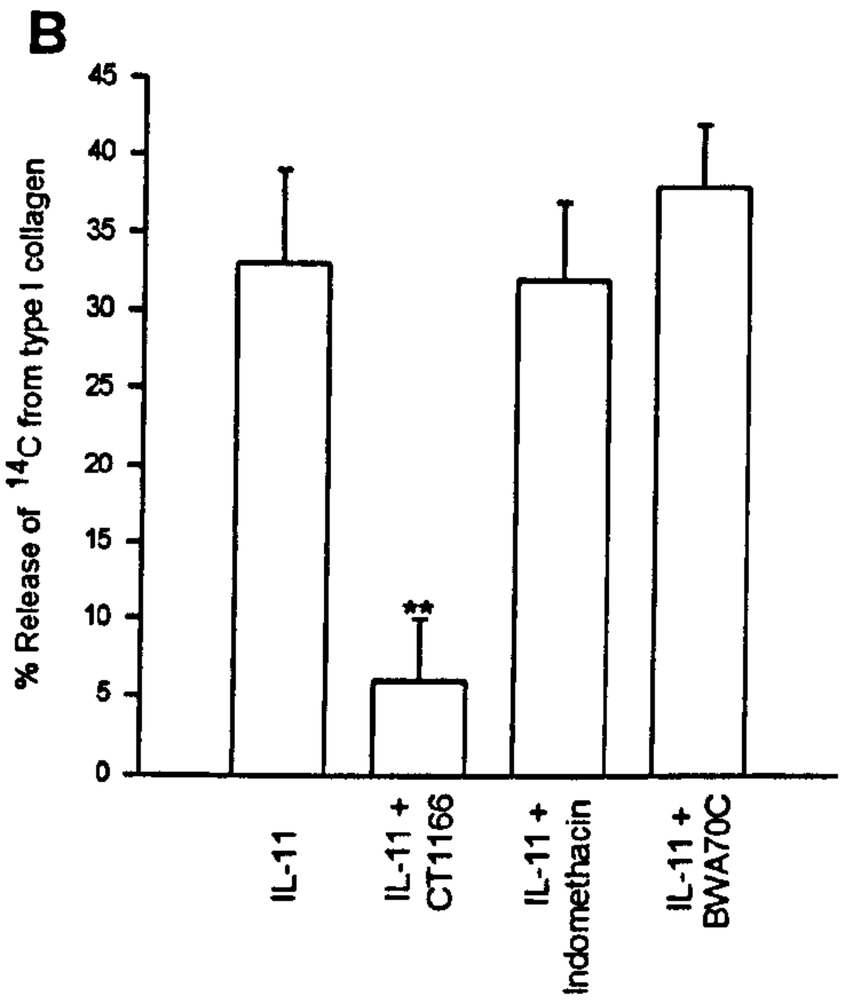


FIG. 4-2 Effects of IL-11 and 1,25-$(OH)_2D_3$ on the degradation of ^{14}C-labelled type-I collagen films by mouse osteoblasts.

A. Mouse osteoblasts were cultured at a density of 10^5 cells/well of a 24-well plate in the presence of increasing concentrations of either IL-11 or 1,25-$(OH)_2D_3$ for 96 h. The results are the mean ± SEM of 6 separate cultures. The percentage release of ^{14}C from the control (unstimulated) cultures was 24.1 ± 4.9. **B.** Effects of inhibition of MMP, PG or LT synthesis on the degradation of ^{14}C-labelled type-I collagen films by mouse osteoblasts stimulated by either IL-11 or 1,25-$(OH)_2D_3$. Mouse osteoblasts were cultured as in **A.** in the presence of either IL-11 (10^{-9} M) or 1,25-$(OH)_2D_3$ (10^{-8} M) for 96 h. The various ligands were added 6 h after sedimentation of the osteoblasts. The inhibitory effects of CT1166 (10^{-6} M) were statistically significant, $^{**}P<0.01$, compared with the control whilst the inhibitory effects of indomethacin (10^{-6} M), BWA70C (10^{-6} M) and MK886 (10^{-6} M) were not statistically significant compared to the control.

TABLE 4-1. Effects of IL-11 and 1,25-$(OH)_2D_3$ on lacunar resorption by isolated osteoclasts and by osteoclasts cocultured with osteoblasts.

Treatment	No. of pits/ivory slice	Surface area of resorption/ ivory slice (μm^2)
OC	16.7 ± 3.1	4186 ± 815
OC + IL-11	19.2 ± 4.5	4214 ± 971
OC + 1,25-$(OH)_2D_3$	20.3 ± 4.2	4695 ± 761
OC + OB	43.7 ± 9.7	11,034 ± 2016
OC + OB + IL-11	47.2 ± 11.3	10,709 ± 3169
OC + OB + 1,25-$(OH)_2D_3$	175.0 ± 30.1	63,616 ± 12132

Mouse osteoclasts (OC) were sedimented onto ivory slices for 25 min and cultured alone or in the presence of mouse osteoblasts (OB). IL-11 and 1,25-$(OH)_2D_3$ were added to the cultures once the cells had attached to the substrate. After incubation for 48 h in the presence of either IL-11 (10^{-9} M) or 1,25-$(OH)_2D_3$ (10^{-8} M), the resorption was quantified by image analysis. Each mean ± SEM represents 12 ivory slices from four separate experiments. The number of TRAP positive multinucleated osteoclasts per slice was 24.3 ± 6.2 and the mean surface area of the pits was 305.7 ± 125 μm^2.

4.3.4 Effects of IL-11 on TRAP-positive MNC formation in bone marrow cultures

To determine the possible involvement of IL-11 in the regulation of TRAP-positive MNC generation, IL-11 was added to the cultures separately or in combination with 1,25-$(OH)_2D_3$. In the absence of 1,25-$(OH)_2D_3$, IL-11 dose-dependently increased the formation of TRAP-positive MNC (Fig. 4-3A). The EC_{50} for IL-11 effects on TRAP-positive MNCs was 3×10^{-11} M. Maximal TRAP-positive MNC formation occurred at a concentration of 10^{-9} M. The numbers of TRAP-positive MNC formed in 16 independent cultures treated with IL-11 (10^{-9} M) was 136.7 ± 22.3 (Fig. 4-3A). IL-11 was not as effective as 1,25-$(OH)_2D_3$ (10^{-8} M) in generating TRAP-positive MNC (179.7 ± 27.8). However, IL-11 had an additive effect on TRAP-positive MNC formation when added in combination with 1,25-$(OH)_2D_3$ (226.6 ± 36.7).

4.3.5 Effects of IL-11 on bone resorption in marrow cultures

The cultures in which TRAP-positive MNC were generated in the presence of IL-11 had the ability to form resorption pits when cultured on an ivory substratum (Fig 4-4). Since IL-11 did not enhance the resorptive activity of mature osteoclasts compared to unstimulated cultures (Table 4-1) this indicates that the IL-11-mediated resorption in the marrow cultures reflects the effects of IL-11 on osteoclast formation. Therefore the effects of IL-11 on resorption in the marrow cultures were quantified. When marrow mononuclear cells were cultured on ivory slices for 8 days, IL-11 induced a dose-dependent increase in the surface area of resorption compared with the control cultures (Fig. 4-3B). The EC_{50} for the effects of IL-11 on lacunar resorption was 2×10^{-11} M and a maximal effect occurred at a concentration of 10^{-9} M (Fig. 4-3B). The 5-LO inhibitors, BWA70C and MK886 produced a dose-dependent inhibition in IL-11-mediated resorption with a maximum of ~50% at 10^{-6} M (Fig 4-3C). The PG synthesis inhibitor, indomethacin produced a greater inhibition with a maximum of 83.6 ± 9.7% at the same concentration (Fig. 4-3C). Complete inhibition was only achieved using a combination of both types of inhibitor. The MMP inhibitor, CT1166 (10^{-6} M) produced a 28.9 ± 8.7% inhibition in the resorptive activity of the TRAP-postive MNCs (Fig. 4-3C).

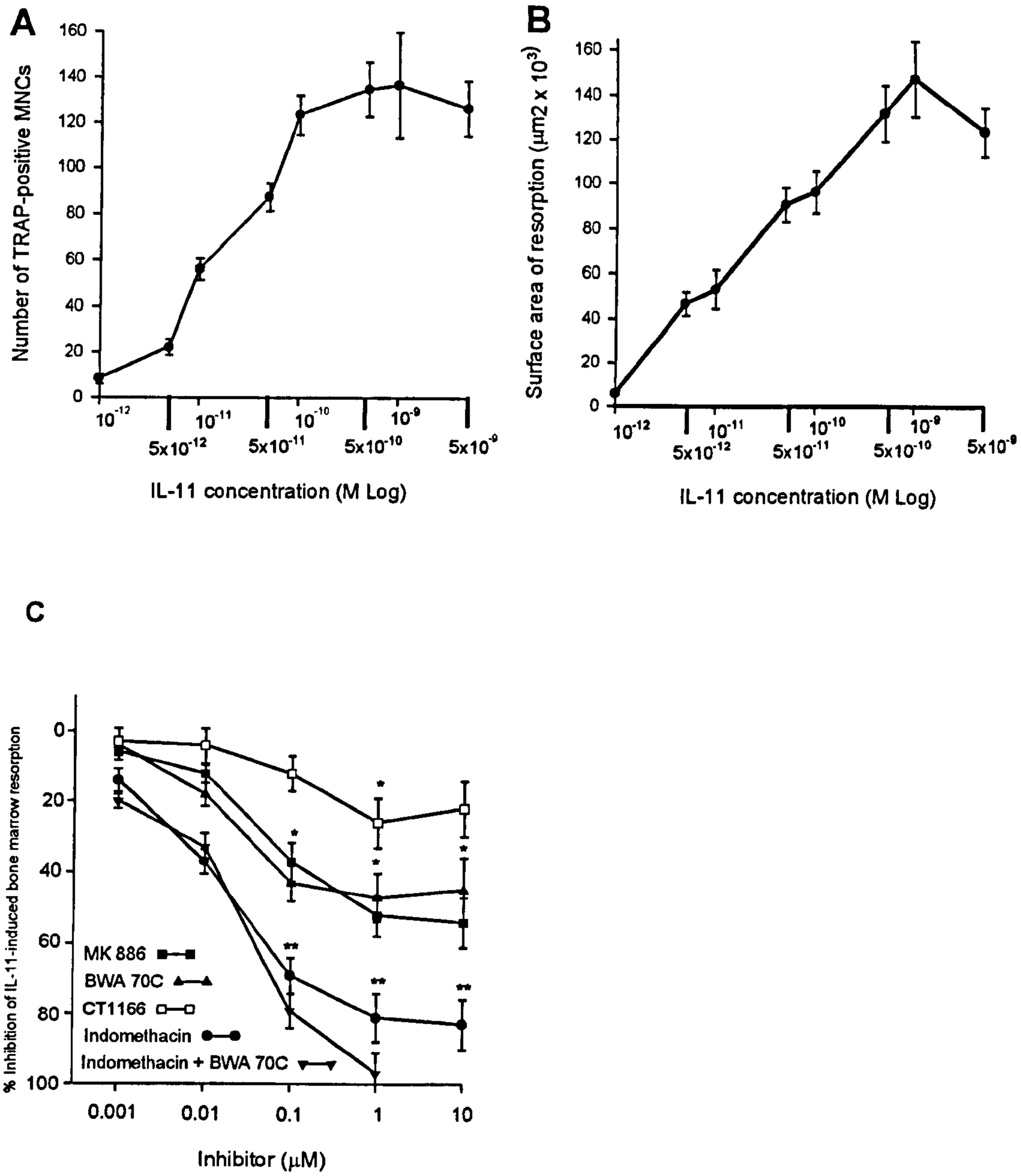


FIG. 4-3 Effects of IL-11 on the formation of TRAP-positive MNCs.
A. Mouse marrow cells were cultured with increasing concentrations of IL-11 for 8 days. Data are expressed as the mean ± SEM of 6 cultures from 2 experiments. **B.** Effects of IL-11 on bone resorption. Bone marrow cells were cultured on ivory slices for 10 days in the presence of increasing concentrations of IL-11. Lacunar resorption was quantified by image analysis. Data are expressed as the mean ± SEM of four cultures from two experiments. **C.** Effects of inhibition of PG, LT and MMP synthesis on IL-11 stimulated lacunar resorption. Murine bone marrow cells were cultured on ivory slices for 10 days and stimulated by IL-11 (10^{-9} M). IL-11, CT1166, BWA70C and MK886 were added to the cultures 8 h after sedimentation of the marrow cells. The inhibitory effects of CT1166, BWA70C, MK886, and indomethacin were statistically significant, *, $P<0.05$; **, P 0.01 compared with the control. Lacunar resorption was quantified by measuring the surface area of the lacunae by Image Analysis. Data are expressed as the mean ± SEM of four cultures from 2 experiments.

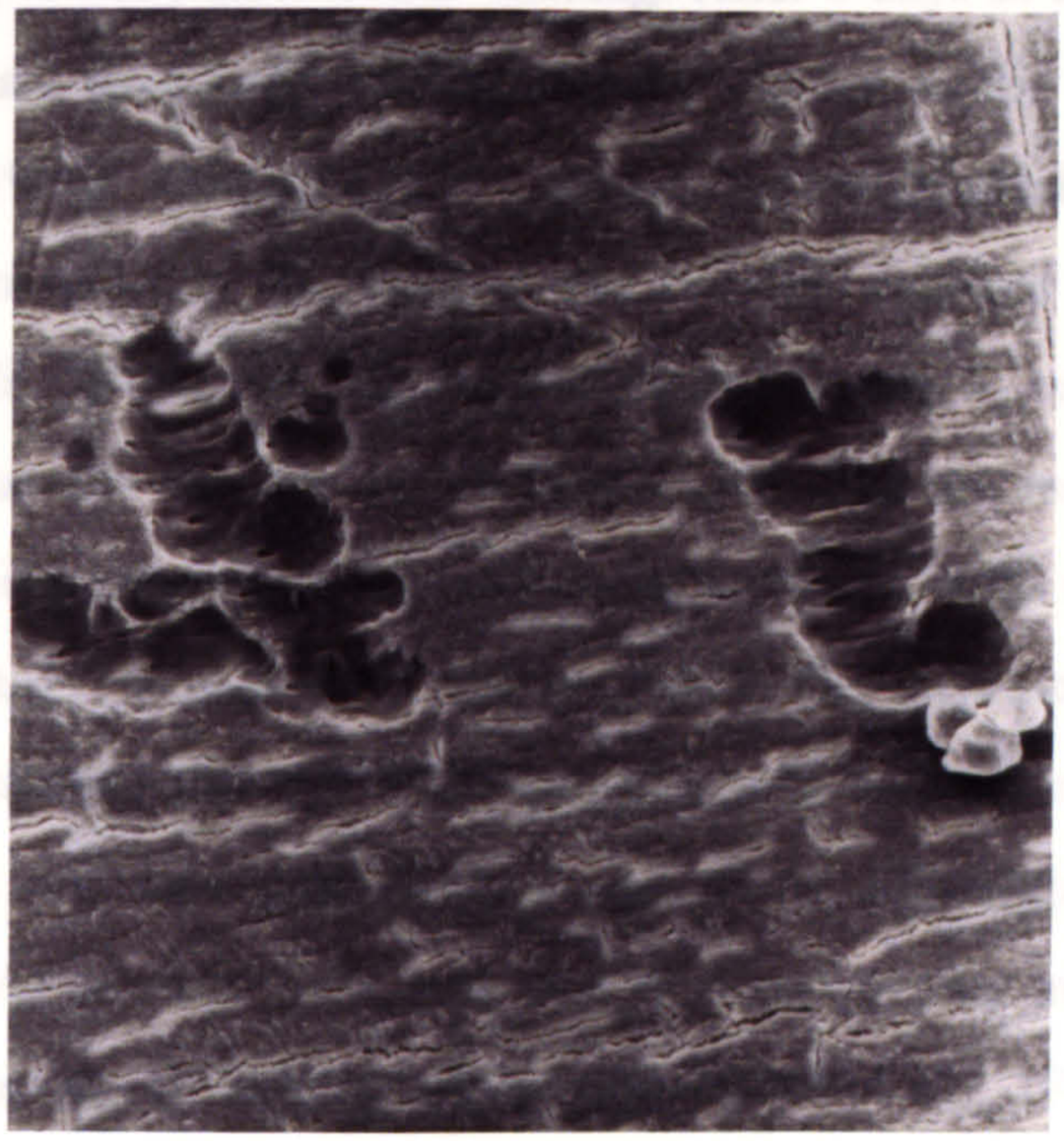


Fig. 4-4 Scanning electron micrograph of resorption pits.
Bone marrow cells were cultured on ivory slices and cultured in the presence of IL-11 as described in Materials and Methods. After 8 days ivory slices were removed and processed for scanning electron microscopy. Slices show large resorption lacunae. Magnification X 900.

4.3.6 Effects of IL-11 on osteoclast migration and fusion in 17-day-old metatarsal rudiments

The effects of IL-11 and 1,25-$(OH)_2D_3$ on osteoclast migration and fusion were investigated in non-invaded 17-day-old metatarsal bone rudiments after 3 days of culture by means of histomorphometry after TRAP staining. After 3 days of culture a maximal number of mature osteoclasts have been formed and are present in the primitive marrow cavity (Scheven *et al.*, 1986). Culturing for a longer period gradually diminishes osteoclast numbers, which is most likely the result of less available calcified matrix due to resorption (Baron *et al.*, 1986). When the cultures are started at day 0, cell and nuclei counts are similar, showing that the majority of TRAP-positive cells are mononucleated (Fig. 4-5). Upon culture there is a progressive increase in the number of cells and nuclei per metatarsal as described previously (Blavier and Delaisse, 1995). The increase is larger for nuclei than for cell numbers, showing that the TRAP-positive cells become multinucleated (Fig. 4-5). These observations are compatible with a continuous differentiation of TRAP-negative precursors into TRAP-positive cells, and with the concept that multinucleated osteoclasts are generated by fusion of TRAP-positive cells (Baron *et al.*, 1986). Fig. 4-5 shows the effect of 3 days of continuous treatment with either IL-11 (10^{-9} M) or 1,25-$(OH)_2D_3$ (10^{-8} M) on the migration kinetics of the maturing osteoclasts to the mineralized matrix, as evaluated from the counts of the total number of TRAP-positive cells and their nuclei in serial sections of a number of metatarsals. In the 1,25-$(OH)_2D_3$ treated cultures there is a significantly greater increase in the number of TRAP-positive cells and nuclei per metatarsal as compared to the control cultures. Culture with 1,25-$(OH)_2D_3$ leads also to an increase numbers of TRAP-positive cells and of nuclei in the mineralized matrix; the proportion of nuclei in the matrix increases from 0 to 43 for the control cultures and from 0 to 116 for the 1,25-$(OH)_2D_3$ treated cultures (Fig. 4-5). In contrast, although IL-11 (10^{-9} M) induced an increase in the number of TRAP-positive cells and their nuclei, the cells were prevented from invading the mineralized matrix (Fig. 4-5). In Fig. 4-6 histological sections of 17-day-old fetal mouse metatarsal are shown after 3 days in culture with and without IL-11. In the IL-11-treated metatarsal explant (Fig. 4-6A) TRAP-positive multinucleated osteoclast precursors (arrows) are still confined periosteum whereas in the untreated explant TRAP-positive cells have invaded the mineralized matrix (Fig. 4-6B).

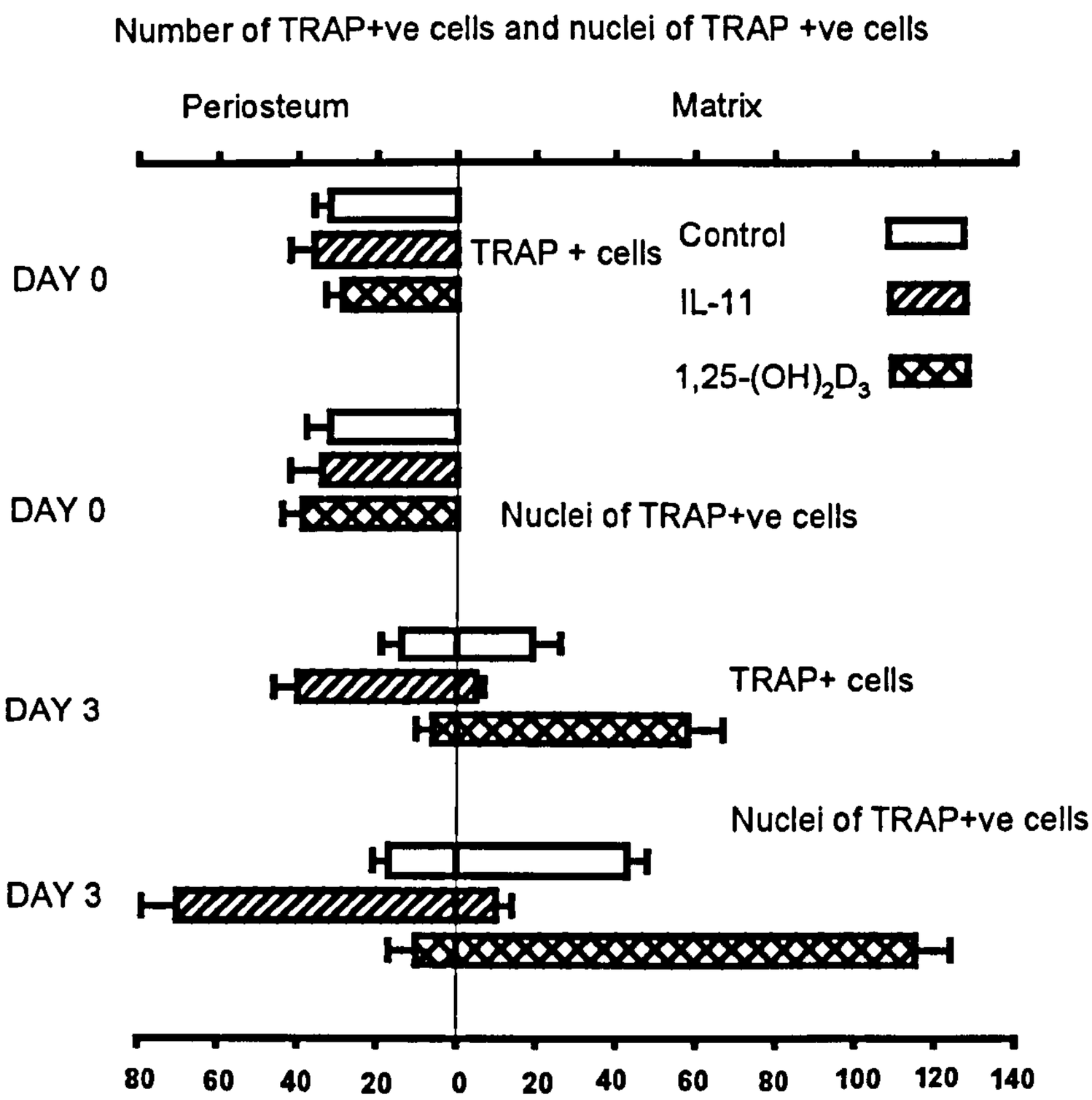


Fig. 4-5. Effects of IL-11 and 1,25-$(OH)_2D_3$ on the migration of TRAP-positive cells in metatarsal explants.

Metatarsals were obtained from 3 litters of 17-day-old fetal mice. The metatarsal triads of the left limb were cultured with either IL-11 (10^{-9} M) or 1,25-$(OH)_2D_3$ (10^{-8} M) and those of the corresponding right limbs were cultured under control conditions for 3 days. The number of TRAP-positive cells and their nuclei localized inside and outside the calcified cartilage (cc) were counted. Counts inside the cc are shown to the right of the '0' axis and those within the periosteum are shown to the left. Each bar (left and right) expresses thus the total number in one metatarsal. Counts at day 0 and 3 are the means ± SEM of 12 and 15 metatarsals respectively.

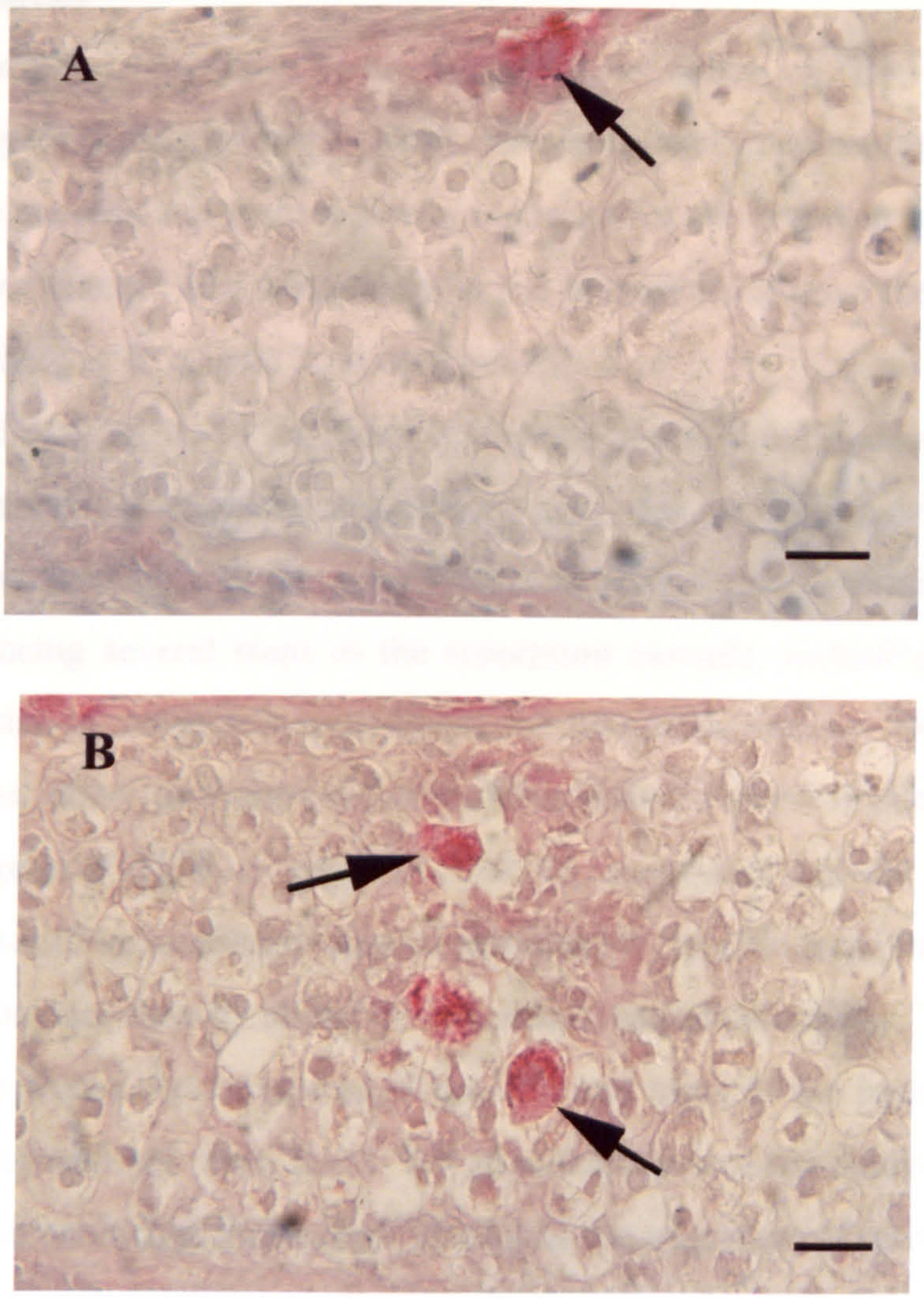


Fig. 4-6. Light micrographs of 17-day-old fetal metatarsal rudiments.
17-day-old fetal metatarsal rudiments were cultured for 3 days in medium with IL-11 (10^{-9} M; A) and control medium (B). In the IL-11 treated rudiment (A), large TRAP-positive cells are present in the periosteum (arrow), but no TRAP-positive cells have invaded the mineralized center, which is not resorbing. In the control rudiment (B), several TRAP-positive cells (arrows) have entered the rudiment. Mineralized matrix is being resorbed in the centre of the bone. Bar = 50 μm.

4.4 DISCUSSION

This investigation looks at the mechanism of action of IL-11 on bone resorption using a variety of discriminatory *in vitro* culture systems representing different aspects of the resorptive cascade. As there is strong evidence for the involvement of both MMPs and products of arachidonic acid metabolism in the resorption of bone their contribution to the resorptive activity of IL-11 has been determined.

The effects of IL-11 on bone resorption were initially assessed using neonatal calvarial explants that comprise a hetereogenous cell population which includes mature osteoclasts and their precursors. These explants enable one to simultaneously screen for activity influencing several steps in the resorption cascade, including osteoblast-mediated osteoid degradation, osteoclast recruitment and osteoclast activity. Demonstration that IL-11 was without effect on bone resorption for the first 2 days of culture compared to the osteotropic agents PTH, IL-1 and 1,25-$(OH)_2D_3$ suggested that the mechanism by which IL-11 modulates bone resorption may be restricted to specific steps in the process and that the cytokine may not directly stimulate osteoclast resorptive activity. This was investigated using selective assays representative of the different steps in the bone resorption cascade. The study demonstrates that IL-11 (i) stimulates osteoblast-mediated type I collagen degradation and osteoclast formation, (ii) inhibits the migration of (pre)osteoclasts to future resorption sites and (iii) has no effect on the activity of mature osteoclasts. Furthermore, the use of selective inhibitors has shown that the CO and 5-LO products of arachidonic acid metabolism play a part in the osteoclastogenic activity of IL-11 and that MMPs are involved in mediating osteoblast degradation of the unmineralized osteoid layer of bone in response to IL-11.

Cytokines exert their pleiotropic effects by interacting with specific cell surface receptors (Kishimoto *et al.*, 1994). Osteoblasts express transcripts for the complete IL-11 receptor which consists of two components: a unique ligand-binding chain (IL-11Rα) (Yin *et al.*, 1992) and a non-ligand binding, signal transducing chain (gp 130) (Yin *et al.*, 1993). The results with the selective MMP inhibitor, CT1166, indicate that the interaction of IL-11 with its receptors on osteoblasts enhances the synthesis of MMPs that are responsible for the degradation of type I collagen. This upregulation in osteoblast MMP production is similar to that induced by several osteotropic factors, including IL-1, PTH and 1,25-$(OH)_2D_3$, which also cause osteoblasts to degrade type I collagen (Hill *et al.*, 1995; Meikle

et al., 1992). Therefore, it appears that the increase in MMP activity is a common denominator among the bone-resorbing actions of several agents. Osteoblasts are a source of IL-11 (Elias *et al.*, 1995; Romas *et al.*, 1996) and upon stimulation by several osteotropic agents, including PTH, 1,25-$(OH)_2D_3$, and IL-1, the expression of IL-11 and the signal transducing component of the IL-11 receptor, gp130 in osteoblasts is enhanced (Romas *et al.*, 1996). These observations suggest that IL-11 may play a central role in osteoblast-mediated type I collagen degradation and that the regulation of gp130 by certain osteotropic agents may modulate the sensitivity of osteoblasts to IL-11.

Although IL-11 receptor transcripts have been demonstrated in mature osteoclasts (Romas *et al.*, 1996), the inability of IL-11 to stimulate bone resorption even when osteoclasts were cocultured with osteoblasts suggests that the IL-11 receptor is not involved in this aspect of osteoclast activity. However, the demonstration that IL-11 stimulates osteoclast formation and prevents the migration of (pre)osteoclasts to resorption sites suggests that the receptor may play a role in these aspects of the resorption cascade. IL-11 has recently been shown to increase expression of RANKL in cultures of primary mouse osteoblasts which explain the effects of IL-11 on osteoclast generation (Horwood *et al.*, 1998).

PGs are produced in bone by many cells, especially osteoblasts, and production is stimulated by a variety of cytokines derived from macrophages or hemopoietic cells within the bone marrow microenvironment (Feyan *et al.*, 1984). In the present study an important role for PGs in IL-11-mediated osteoclast formation has been demonstrated which confirms a recent report (Girasole *et al.*, 1994). However based upon the effects of two selective lipoxygenase inhibitors, BWA70C and MK886 the findings from this study indicate that lipoxygenases also contribute to the osteoclastogenic activity of IL-11. The IC_{50} values for the inhibitors are in accord with the reported potency of these compounds and so the results would appear to be due to the selective inhibitory activity of the compounds and not to non-specific effects. The fact that two mechanistically distinct classes of lipoxygenase inhibitor can block bone resorption induced by IL-11 reinforces the fact that these enzymes are induced by this particular osteolytic cytokine. At present it is not certain as to which particular lipoxygenase products of arachidonic acid metabolism are responsible for mediating the effects of IL-11 on osteoclast formation due to the instability and short half-life of the products (Keppler, 1992). Interestingly, a role for leukotriene B_4 in

osteoclastogenesis has recently been shown in murine bone marrow cultures (Garcia *et al.*, 1996). Whilst PGs have been shown to mediate the bone resorptive activity of a variety of cytokines, this is the first indication that the 5-LO products of arachidonic acid metabolism might also play a role in the osteolytic activity of a pleiotropic cytokine.

The ability of IL-11 to induce osteoclast development when added to cultures of bone marrow cells by itself contrasts with the related cytokine, IL-6 whose biological activities are also mediated by the gp130 signal transducer (Taga *et al.*, 1989). IL-6 induction of osteoclast differentiation, however, is dependent on the presence of soluble IL-6 receptors (Tamura *et al.*, 1993) and is mediated by IL-6 receptors expressed on osteoblastic cells rather than osteoclast progenitors (Udagawa *et al.*, 1995). The ability of IL-11 to induce osteoclast differentiation on its own may be due to the presence of IL-11 receptors on osteoclasts or because osteoblasts express a sufficient levels of functional IL-11 receptors.

Osteoclast formation is induced by at least three different mechanisms (Suda *et al.*, 1992). The first mechanism is the PTH, IL-1, PGE_2 axis which is mediated by signaling involving cAMP. The second mechanism is 1,25-$(OH)_2D_3$ induced osteoclast formation, which is mediated by the vitamin D receptor but independent of cAMP. The gp130 signal, activated by cytokines such as IL-11, IL-6 and LIF is an additional and important pathway of osteoclast formation. Interestingly IL-11 may contribute in part to osteoclast formation induced by some osteotropic agents because antibodies to either gp130 or IL-11 inhibit the osteoclastogenic effects of PTH, IL-1, PGE_2, and 1,25-$(OH)_2D_3$ (Romas *et al.*, 1996; Girasole *et al.*, 1994). Furthermore these agents stimulate IL-11 production in cocultures of osteoblasts and bone marrow cell cultures (Romas *et al.*, 1996; Girasole *et al.*, 1994).

In this study it has been shown that 1,25-$(OH)_2D_3$ increases the number of TRAP-positive cells that migrate from the periosteum to mineralized matrix in the center of the rudiments where they excavate a primitive marrow cavity. 1,25-$(OH)_2D_3$ increased the number of TRAP cells in the bone center as well as the excavation of a marrow cavity. Both phenomena, accumulation of cells in the resorbing center and cell fusion imply cell movement. In contrast IL-11 prevented the migration of TRAP-positive osteoclasts to future resorption sites without effecting their fusion into multinucleated cells. An accumulation of TRAP-positive cells in the periosteum accompanies the blockage of migration, thus indicating that the generation of TRAP-positive cells is not prevented. The

failure of (pre)osteoclast invasion of the mineralized matrix cannot be ascribed to an antiproliferative effect of IL-11 as the cells responsible for resorption have already reached a post-proliferative stage at the onset of the cultures, as shown by irradiation (Scheven *et al.*, 1986).

It is highly unlikely that PG synthesis is involved in the inhibitory effects of IL-11 on osteoclast migration, since PGs stimulate resorption in fetal long bones, which implies that osteoclast migration is enhanced by PGs. The inhibitory activity of IL-11 on (pre)osteoclast migration may be due to an alteration in the balance between MMPs and their natural inhibitors, the tissue inhibitor of metalloproteinases (TIMPs), as some modulators of this balance, such as TNF-α, TGF-β and LIF (Murphy and Reynolds, 1993) have been found to interfere with the migration of (pre)osteoclasts into the mineralized matrix of metatarsal rudiments (Van der Pluijm *et al.*, 1991; Dieudonne *et al.*, 1991; Van Beek *et al.*, 1993). In support of this concept it has recently been demonstrated that an MMP inhibitor prevented the migration of (pre)osteoclasts induced by 1,25-$(OH)_2D_3$ and that migrating osteoclasts express gelatinase B (MMP-9) (Blavier and Delaisse, 1995), a proteinase involved in several processes where cells invade connective tissues, including wound healing, ovulation, tumour invasion and metastases. Furthermore high levels of TIMP have been detected in the periosteum of developing bones (Nomura *et al.*, 1989) and within isolated osteoclasts (Hill *et al.*, 1994). Thus TIMP may restrict the migration of (pre)osteoclasts directly or indireclty by limiting the lysis of the periosteum to focal points. Interestingly IL-11 stimulates the production of TIMP in chondrocytes and synoviocytes thereby limiting connective tissue degradation (Maier *et al.*, 1993).

5. Expression and Function of Members of the ADAMs Family of Proteins in Bone Cells.

5.1 INTRODUCTION

It is well established that multinucleated osteoclasts are formed by fusion of mononuclear osteoclast precursors derived from hematopoietic progenitor cells (Nijweide *et al.*, 1986) and that osteoclastogenesis critically involves cell-cell interactions between osteoclast precursors and osteoblasts (Takahashi *et al.*, 1988). The precise repertoire of proteins and cellular mechanisms involved in these cell-cell interactions and subsequent events leading to cellular fusion are not fully elucidated. A role for integrins and non-integrin cellular adhesion molecules in the processes leading to osteoclast formation has been well established. E-cadherin is expressed by mouse and human osteoclasts and neutralization of E-cadherin function decreases the number of osteoclasts formed in bone marrow cultures (Mbadaviele *et al.*, 1995). Furthermore, synthetic RGD peptides have been shown to inhibit fusion of mononuclear osteoclast precursors (van der Pluijm *et al.*, 1994) thus implicating the integrin class of adhesion molecules in osteoclast formation.

Recently, members of the ADAM family of membrane bound glycoproteins have been cloned, characterized and shown to have a multiple domain structure consisting of a metalloproteinase domain, a disintegrin-like domain, a cysteine rich domain, a transmembrane region and an intracellular domain (Wolfsberg and White, 1996). To date 30 members of this family have been isolated and implicated in a number of important cell-cell and cell-matrix interactions. ADAM-15 (metargidin) has been shown to interact via its disintegrin domain with the $\alpha_v\beta_3$ integrin expressed on chinese hamster ovary cell lines (Zhang *et al.*, 1998) and with the $\alpha_v\beta_1$ integrin on haemopoietic cells (Nath *et al.*, 1999). The cysteine rich domain of recombinant human ADAM-12 has also been shown to promote cellular adhesion in a number of human tumour cell lines (Iba *et al.*, 1999), whilst the disintegrin domain of ADAM-12 has been found to interact with the $\alpha_9\beta_1$ integrin in an RGD-independent manner (Eto *et al.*, 2000).

The cysteine rich domains of ADAM-1 (fertilin α) and ADAM-12 contain a short hydrophobic stretch of approximately 30 amino acids, which bears homology to fusion peptides present in viruses. ADAM-1 forms a heterodimer with ADAM-2 (fertilin β) and

has been shown, using blocking antibodies and synthetic peptides, to be involved in sperm-egg binding and subsequent fusion (Myles *et al.*, 1994; Almeida *et al.*, 1995). Yagami-Hiromasa *et al.* (1995) cloned ADAM-12 (meltrin-α) from C2C12 muscle cells and demonstrated using antisense RNA that ADAM-12 is involved in myoblast fusion. In addition ADAM-9 (meltrin-γ) and ADAM-19 (meltrin-β) were cloned and may be involved in fusion events. ADAM-12 and ADAM-19 have been shown to be expressed in osteoblastic cells and it has been speculated that they may be involved in osteoblast differentiation (Inoue *et al.*, 1998).

The aims of this study were firstly to investigate the expression of ADAMs-9, -12 and -19 in (1) primary mouse osteoblasts by RT-PCR and (2) in isolated osteoclasts and in the 17-day-old embryonic mouse metatarsal model by *in situ* hybridization. Secondly, to assess the function of ADAM-12 using bioassays of osteoclast formation and osteoblast differentiation.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Recombinant human RANKL was from Insight Biotechnology Ltd. (Middlesex, UK) and recombinant murine M-CSF was from R & D Systems Ltd. (Abingdon, Oxon, UK). Recombinant glutathione-S-transferase (GST) and all chemicals and cell culture reagents were purchased from Sigma. CD-1 mice were purchased from Charles Rivers. The plasmid pGEX-2T was a generous gift from Dr. A. Waseem (Department of Craniofacial development, Guy's hospital) and the plasmids pcDNA-3 and pSPAP (secreted placental alkaline phosphatase) were obtained from Dr. A. Sunters (Craniofacial development, Guy's hospital).

5.2.2 Methods

5.2.2.1 Extraction of total RNA

Total RNA was prepared from osteoblasts using an RNA isolation kit (Stratagene, UK) according to the manufacturer's instructions. Briefly RNA was isolated using acid guanidinium thiocyanate-phenol chloroform extraction and precipitated using isopropanol

and sodium acetate (0.2 M; pH 4.0). Total RNA was recovered by centrifugation at 10000 x g for 20 min at 4°C, washed with 75% ethanol, and dissolved in nuclease-free water.

5.2.2.2 RT-PCR procedure

Oligonucleotide PCR primers were designed using Designer PCR (Research Genetics, UK) from previously published sequence data shown in Table 6-1 and synthesized by Life Technologies, UK.

Table 5-1. Oligonucleotide RT-PCR primer sequences for mouse ADAMs

		Primer Sequence	Expected Product Length (base pair)
ADAM 9	F	**5'-ACAGCGAAGGAGTGTGAGGTGG-3'**	**188**
	R	**5'-GGGCAGAACTGAGAGGAACCG-3'**	
ADAM 12	F	**5'-CCTGGTGAGCATCCTGTGTCTG-3'**	**241**
	R	**5'-GCATTTCAGCGAGTGCCTGTC-3'**	
ADAM 19	F	**5'-GAGGAGGAAGGGGAAGGTGAC-3'**	**189**
	R	**5'-GCCAGGGAAGCAATGACAGTT-3'**	
GAPDH	F	**5'-CCACGAGAAATATGACAAC-3'**	**222**
	R	**5'-GATGCAGGGATGATGTTC-3'**	
β-ACTIN	F	**5'-CAGGCATTGTGATGGACTCCG-3'**	**186**
	R	**5'-CACGCACGATTTCCCTCTCAG-3'**	

RT-PCR reactions were performed using the GeneAmp *rTth* Reverse Transcriptase RNA PCR Kit (Perkin Elmer, USA) and a Stratagene Robocycler Gradient 96 PCR machine (Stratagene, UK). Briefly, 20 µl reverse transcription (RT) reactions were prepared from a master mix consisting of 1.0 mM $MnCl_2$, 200 µM each of dGTP, dATP, dTTP and dCTP and 5 units of *rTth*. To each 20 µl RT reaction was added 250 ng of total RNA and reverse primer (15 pmol). Each reaction was overlaid with mineral oil and incubated at 70°C for 15 min. Following RT the reactions were held at 4°C and 100 µl PCR-reactions

prepared by addition of a master mix consisting of chelating buffer and $MgCl_2$ (final concentration 1.5 mM). To each 100 µl PCR reaction was added the matching forward primer (15 pmol) and the reaction overlaid with mineral oil. PCR mixtures were incubated at 95°C for 3 min followed by 35 cycles at 94°C (melting temperature) for 1.0 min, 55°C (annealing temperature) for 1.5 min and 72°C (primer extension) for 1.0 min. A final step was performed at 94°C for 1.0 min, 55°C for 1.5 min and 72°C for 10.0 min. RT-PCR products were analyzed by electrophoresis on a 2.0% agarose gel in Tris-Acetate-EDTA buffer (TAE) containing ethidium bromide (0.5 µg/ml). Band size was checked against molecular weight standards (ØX174 Hae III digest).

5.2.2.3 Cloning of RT-PCR products in pGEM-T

RT-PCR products were electrophoresed on 1.5% low melting point agarose gels in Tris-Acetate-EDTA (TAE; Appendix 1). The expected DNA fragments were excised and purified from a TAE gel using QIAquick gel extraction kit (Qiagen GmbH, Germany). The authenticity of the sequences was verified by automated sequencing (Advanced Biotechnology Centre, Imperial College of Science, Technology and Medicine, UK). The fresh PCR products were ligated to pGEM-T vector (Promega, Southampton, UK) using T4 DNA ligase (Promega), and then transformed to high efficiency JM109 *E.coli*. Recombinant clones were selected for on Luria-Bertani (LB) medium containing ampicillin (100 µg/ml), isopropylthio-β-D-galactoside (0.5 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; 80 µg/ml). *E. coli* were cultured overnight in 250 ml of LB broth containing ampicillin (100 µg/ml) and plasmid DNA was extracted using a method based on Qiagen Maxiprep DNA purification (Qiagen). Plasmids were linearized with NcoI and SpeI for synthesis of Digoxigenin-11-UTP labelled riboprobes.

5.2.2.4 Preparation of MMP-9 probe

The probe for MMP-9 was a generous gift from Karl Tryggvason (Karolinska Institute, Sweden) and consisted of a 323 base pair fragment cloned into the Sma I/EcoRI site of plasmids pSP64/65. The plasmids were transformed to competent JM109 *E. coli* and recombinant clones selected for on LB medium containing ampicillin (100 µg/ml). *E. coli* were then cultured in 250 ml of LB broth containing ampicillin (100 µg/ml) and plasmid

DNA extracted using a method based on Qiagen Maxiprep DNA purification (Qiagen). Plasmids were linearized with BamH1 for synthesis of Digoxigenin-11-UTP riboprobes.

5.2.2.5 Preparation of riboprobes

For *in situ* hybridization, digoxigenin-11-UTP labelled sense and antisense riboprobes were synthesized from linearized template plasmids using a DIG RNA labelling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The sense RNA strand was used as a negative control for each probe. Before use riboprobes were heated at 85 °C for 5 min.

5.2.2.6 In situ hybridization

Paraformaldehyde (PFA)-fixed sections were deparaffinized in histoclear and rehydrated through a graded series of alcohols. The slides were postfixed in fresh 4% PFA, washed in phosphate buffered saline (PBS), 0.1 M HCl, PBS, distilled H_2O, then Tris buffer/$CaCl_2$ (20 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$). The slides were incubated with 10 µg/ml proteinase K in the same buffer for 15 min and washed with PBS. After washing, postfixation was carried out in 4% PFA for 10 min. Further washes in PBS and 0.1 M triethanolamine (pH 8.0) were followed by acetylation in 0.25% acetic acid, 0.1 M triethanolamine (pH 8.0) for 20 min. Slides were then washed in PBS. Prehybridization was performed using hybridization buffer (50% formamide, 5X SSC(0.3 M NaCl and 0.03 M sodium citrate), 5X Denhardt's solution, 250 µg/ml tRNA) for 3 h at room temperature. Hybridization was carried out under coverslips using 50 µl of hybridization solution with the digoxigenin-labelled probe and incubated at 50°C overnight. Excess probe was removed by incubating in 5X SSC for 5 min at 50°C, 0.2X SSC for 1 h at 50°C and 100 mM Tris HCl pH 7.5, 150 mM NaCl for 5 min at room temperature. Slides were incubated with 10% normal sheep serum for 1 h at room temperature and detection performed using alkaline phosphatase labelled sheep anti-DIG Fab fragments (Boehringer Mannheim). Unbound antibody was removed by washing in 100 mM Tris HCl pH 7.5, 150 mM NaCl for 15 min and signal detected by incubating in 50 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ buffer containing nitroblue tetrazolium (340 µg/ml), 5-bromo-4-chloro-3-indoyl phosphate (175 µg/ml) and levamisole (1 mM) overnight. The reaction was stopped by washing with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

5.2.2.7 Oligodeoxynucleotides (ODNs)

The ODNs used for antisense studies were purchased from Biognostik (Göttlingen, Germany) and were phosphorothioated and purified by HPLC. The sequences, corresponding to the first 24 bases from the methionine start site, were:GCGCCGCGCCGG GCGCTCTGCCAT (antisense, AS-ODN) and ATGGCAGAGCGCCCGGCGCGGCGC (sense, S-ODN). A scrambled ODN (Scr-ODN) consisting of the same base composition as the antisense ODN had the following sequence: GCCGGCGCCGCGCGTCCGTTACCG. A search of the NCBI BLAST database revealed no homolgy to any other mouse gene. For assessment of uptake of ODN a fluorescene-isothiocyanate labelled ODN (FITC-ODN) having the same base composition as the AS-ODN was added to cultures.

5.2.2.8 Generation of GST/ADAM-12 cysteine rich domain fusion protein

A cDNA corresponding to amino acids 551-657, encoding the cysteine rich domain of mouse ADAM-12, was generated by RT-PCR from total RNA isolated from primary mouse osteoblasts using the following primers: forward primer, 5'-GGATCCCCGTG-TCAGGGCGTGGAT-3' and reverse primer,5'-GGATCCCTTGTGAACGCCGAAGACA-C-3'. The forward primer and reverse primer contained a BamH1 for ligation. The DNA fragment corresponding to the cysteine rich domain was excised and purified from a 2% TAE gel using QIAquick gel extraction kit (Qiagen GmbH, Germany). The cDNA fragment bearing full length ADAM-12 cysteine rich domain was ligated into the BamH1 site of pGEX-2T expression vector using T4 DNA ligase and transformed into the JM109 strain of *E.coli*. Recombinant clones were selected on LB medium as described in section **5.2.2.4**. Plasmids containing the cysteine rich domain of ADAM-12 were subjected to automated sequencing to confirm the authenticity of the sequence and the correct reading frame for expression of the fusion protein. Plasmids were transformed into the BL21 strain of *E.coli*.for production of recombinant protein (A12cys). Synthesis of the glutathione-S-transferase (GST)/ADAM-12 cysteine rich domain fusion protein was induced in a 500 ml culture of BL21 bacteria by addition of fresh IPTG to a concentration of 0.1 mM. After incubation for 4 h at 37°C the bacterial culture was centrifuged at 2000xg and resuspended in 50 ml of tween lysis buffer (TLB; Appendix 1). The fusion protein was extracted by sonication and purified using glutathione sepharose (Amersham Pharmacia) according to the manufacturer's instructions. The concentration of A12cys was quantified using the BCA protein assay (Pierce Chemical Co., Rockford, USA). The quality of the purified protein

was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis SDS-PAGE on a 12% gel followed by Coomassie brilliant blue staining (Appendix 1) .

5.2.2.9 Construction of ADAM-12 antisense constructs

The cDNA fragment bearing full length ADAM-12 cysteine rich domain was ligated into the BamH1 site of pcDNA-3 expression vector using T4 DNA ligase and transformed into the JM109 strain of *E.coli*. The orientation of the insert was confirmed by restriction enzyme digestion with Kpn1. Plasmids containing the insert in the reverse/antisense orientation (AS/A12) and plasmids containing the insert in the forward/sense orientation (S/A12) were used for transfection of primary mouse osteoblasts.

5.2.2.10 Preparation of osteoblasts from neonatal mouse calvariae

Osteoblasts were prepared and characterized as described in section **2.2.2.1**.

5.2.2.11 Osteoblast differentiation assay

Primary mouse osteoblasts were seeded at a density of 2 x 10^4 cells/well of a 24-well plate. After 24 h osteoblasts were cotransfected with either of the following: AS/A12 and pSPAP, S/A12 and pSPAP, pcDNA-3 and pSPAP, pSPAP or mock transfection with no DNA. SPAP can be assayed for in the culture medium and used as a reporter of transfection efficiency (Cullen and Hallim, 1992). Transfections were performed using effectene transfection reagent (Qiagen) according to the manufacturer's instructions. After 24 h the DNA complexes were removed and replaced with 0.5 ml α-MEM + 10% FBS containing 10 mM β-glycerophosphate and 5 μg/ml ascorbic acid. The medium was changed after 3 days and assayed for SPAP (Appendix 1), thereafter medium was changed every 3 days and cultures were maintained for 14 days.

For quantitation of bone nodules von Kossa staining was performed. Cultures were rinsed with cold PBS and fixed in 10% neutral buffered formalin (Appendix 1) for 15 min, rinsed with distilled water and then stained with 2.5% silver nitrate solution for 30 min at room temperature. Culture plates were rinsed with distilled water before the addition of sodium carbonate formaldehyde (5% Na_2CO_3, 20% formaldehyde) for 5 min. After rinsing the nodules were stained for alkaline phosphatase as described in section **2.2.2.1**. The

percent mineralization was assessed by counting the number of occurences of von Kossa-positive (black) nodules where the presence of nodules coincided with intercepts on a grid

5.2.2.12 Generation of osteoclasts

Bone marrow assay

Osteoclasts were generated from the bone marrow of 5- to 6-week-old mice to assess the effects of AS-ODN on osteoclast formation. Bone marrow was isolated as described in section **4.2.2.7** and plated in 24-well plates at a cell density of 1 x 10^6 cells per well in 0.5 ml α-MEM containing 10% FBS and 10^{-8} M 1,25-$(OH)_2D_3$ with or without an ivory slice. ODNs were added at the beginning of culture and medium was changed every two days with addition of ODNs at each medium change. Osteoclast formation was assessed by counting the number of TRAP-positive multinucleated cells with over two nuclei after 8 days in culture.

Spleen cell assay

Spleen cell suspensions were prepared from spleens of 5- to 6-week old CD1 mice by injection of α-MEM containing 10% FBS into the spleens followed by mechanical dissagregation of the remaining tissue with a scalpel blade. The suspension was washed twice by centrifugation at 300xg for 7 min and resuspended in α-MEM containing 10% FBS. Spleen cells were plated in 24-well plates at a cell density of 1 x 10^6 cells per well in 0.5 ml of α-MEM containing 10% FBS, M-CSF (10 ng/ml) and RANKL (40 ng/ml). Cultures were fed every 2 days by replacing 400 µl of culture medium with fresh medium containing 10% FBS, M-CSF and RANKL. After 7 days osteoclast formation was assessed by counting the number of TRAP-positive multinucleated cells with over two nuclei.

Culture of M-CSF selected bone marrow cells

Bone marrow cells were isolated from 5- to 6-week old CD1 mice to assess the effects of recombinant A12cys on osteoclast formation. Bone marrow cells were isolated as described in section **4.2.2.7**. The resulting bone marrow suspension was resuspended in α-MEM containing 10% FBS, and incubated for 24 h in the presence of M-CSF (10 ng/ml) at a density 5 x 10^5 cells/ml in a 75 cm^2 flask. After 24 h the non-adherent population was harvested, washed once by centrifugation at 300xg, and resuspended in α-MEM +10% FBS

containing 10 ng/ml M-CSF and 40 ng/ml RANKL at a concentration of $5x10^5$ cells/ml. This suspension was added ($1x10^5$ cells/well) to the wells of a 48-well plate with or without an ivory slice. To each well was added A12cys, recombinant GST as a control or vehicle.

Isolation of mature osteoclasts

Mature osteoclasts were prepared as described in section **4.2.2.6**. For *in situ* hybridization studies bone marrow suspensions of 6-day-old mice were sedimented onto 22mm diameter glass coverslips coated with rat tail type I collagen 50 μg/ml (Becton Dickinson) and cultured for 3 h at 37°C. Non-adherent cells were washed from the coverslips with α-MEM and the adherent cells fixed with 4% PFA for 10 min. For assessment of lacunar resorption bone marrow suspensions were plated onto ivory slices in 96-well plates and cultured as described in section **4.2.2.6**.

5.3 RESULTS

5.3.1 RT-PCR analysis of total RNA extracted from primary mouse osteoblasts

Oligonucleotide primers specific for ADAM-9, -12 and -19 were used for RT-PCR analysis of gene expression in primary mouse osteoblasts. As depicted in figure 5-1, RT-PCR analysis revealed expression of ADAM-9, -12 and -19 in unstimulated osteoblasts and the RT-PCR products amplified were of the predicted size. To determine whether expression of either ADAM -9, or -12 or -19 is up-regulated by osteotropic hormones, semi-quantitative RT-PCR was performed on osteoblasts stimulated with PTH (10^{-8} M) for 3, 6 and 24 h. As shown in figure 5-1, expression of ADAM-12 appears to be up-regulated by treatment with PTH with a slight increase in expression over 24 h of stimulation. Expression of ADAM-19 also appears to be up-regulated with an increase in expression after 3 h stimulation with PTH. Analysis of ADAM-9 by semiquantitative RT-PCR showed only steady state levels of mRNA expression after stimulation with PTH.

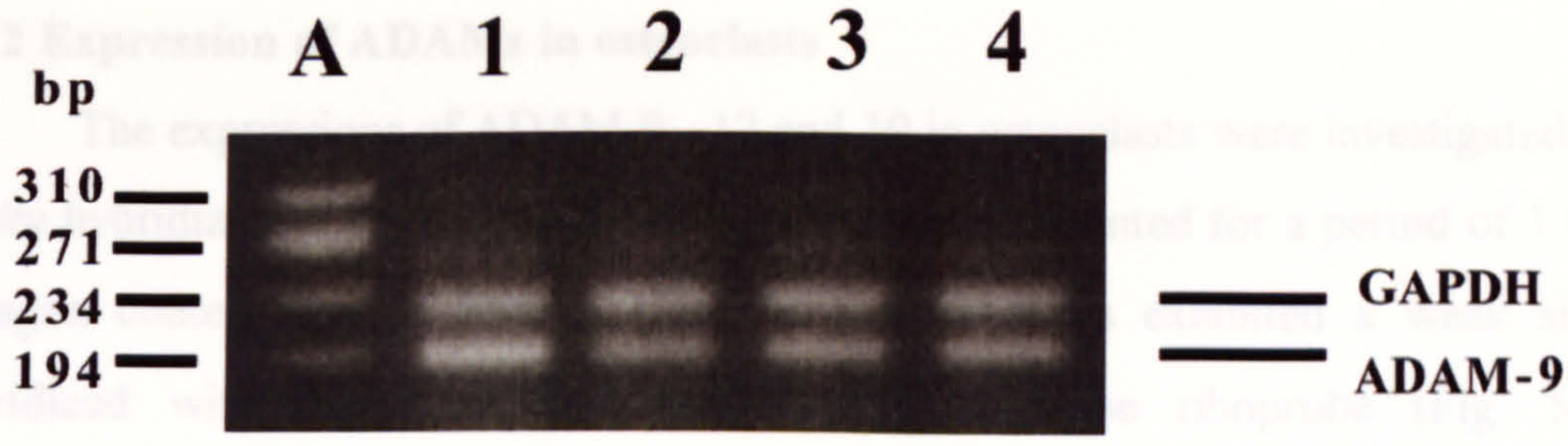


Fig. 5-1. RT-PCR analysis of ADAMs in mouse osteoblasts.
Primary mouse osteoblasts were cultured as described in materials and methods and ttal RNA isolated. RT-PCR was performed with primers specific for ADAM-9, -12 and-19. Each RT-PCR reaction also contained primers for GAPDH (ADAM-9 and -19) or β-Actin (ADAM-12) as a positive control. PCR was performed for 30 cycles and 10 μl analyzed by gel electrophoresis on a 2% agarose gel. Lane **1**, analysis of RNA isolated from unstimulated osteoblasts. Lane **2** RNA isolated from osteoblasts stimulated with PTH for 3 h. Lane **3** RNA isolated from osteoblasts stimulated with PTH for 6 h. Lane 4 RNA isolated from osteoblasts stimulated with PTH for 24 h. **A**, DNA size marker (∅X174 HaeIII digest).

5.3.2 Expression of ADAMs in osteoclasts

The expressions of ADAM-9, -12 and 19 in osteoclasts were investigated initially by *in situ* hybridization studies on isolated osteoclasts sedimented for a period of 3 h on type I collagen coated glass coverslips (Fig. 5-2). Osteoclasts exhibited a weak signal when hybridized with the ADAM-12 DIG-labelled antisense riboprobe (Fig. 5-2C). For comparison osteoclasts hybridized with the corresponding sense riboprobe are shown in figure 5-2D. Expression of ADAMs-9 and -19 were not detected in any of the cultures.

To establish a role for ADAMs in the formation and activity of osteoclasts in 17-day-old fetal mouse metatarsals, *in situ* hybridizations were performed with DIG-labelled riboprobes for ADAM-9, -12 and -19. Metatarsal explants cultured for 3 days in the presence of 1,25-$(OH)_2D_3$ exhibited formation of MMP-9-positive osteoclasts within the calcified cartilage (cc) as shown by *in situ* hybridization (Fig. 5-3G) and subsequent resorption of the cc. mRNA for ADAM-12 was detected in both mononuclear cells and multinucleated osteoclast-like cells within the cc (Fig. 5-3C).

5.3.3 Role of ADAM-12 in osteoblast differentiation

To investigate the role of ADAM-12 in osteoblast differentiation, primary mouse osteoblasts were transiently transfected with either an antisense or sense construct to ADAM-12 in the mammalian expression vector pcDNA-3. Since no antibodies were available to establish whether ADAM-12 protein levels were decreased, the efficiency of the transfection had to be monitored using pSPAP as a reporter. Figure 5-4B shows the presence of SPAP in medium from cultures of osteoblasts that had been cotransfected with the antisense/sense constructs and pSPAP. After 14 days culture in the presence of β-glycerophosphate and ascorbic acid osteoblast cultures had developed large mineralized bone nodules as assessed by the presence of von Kossa positive deposits within the nodules (Fig. 5-4). Cultures also stained strongly for alkaline phosphatase (Fig 5-4). Quantitation of bone nodules expressed as percent mineralization revealed little difference between cultures transfected with AS/A12 (10.0% ± 2.0), S/A12 (11.0% ± 1.4) and mock transfection with no DNA (10.2% ± 1.0). Transfection of cultures with pSPAP alone also had no effect on mineralized nodule formation compared with the mock transfection with no DNA (Fig. 5-4).

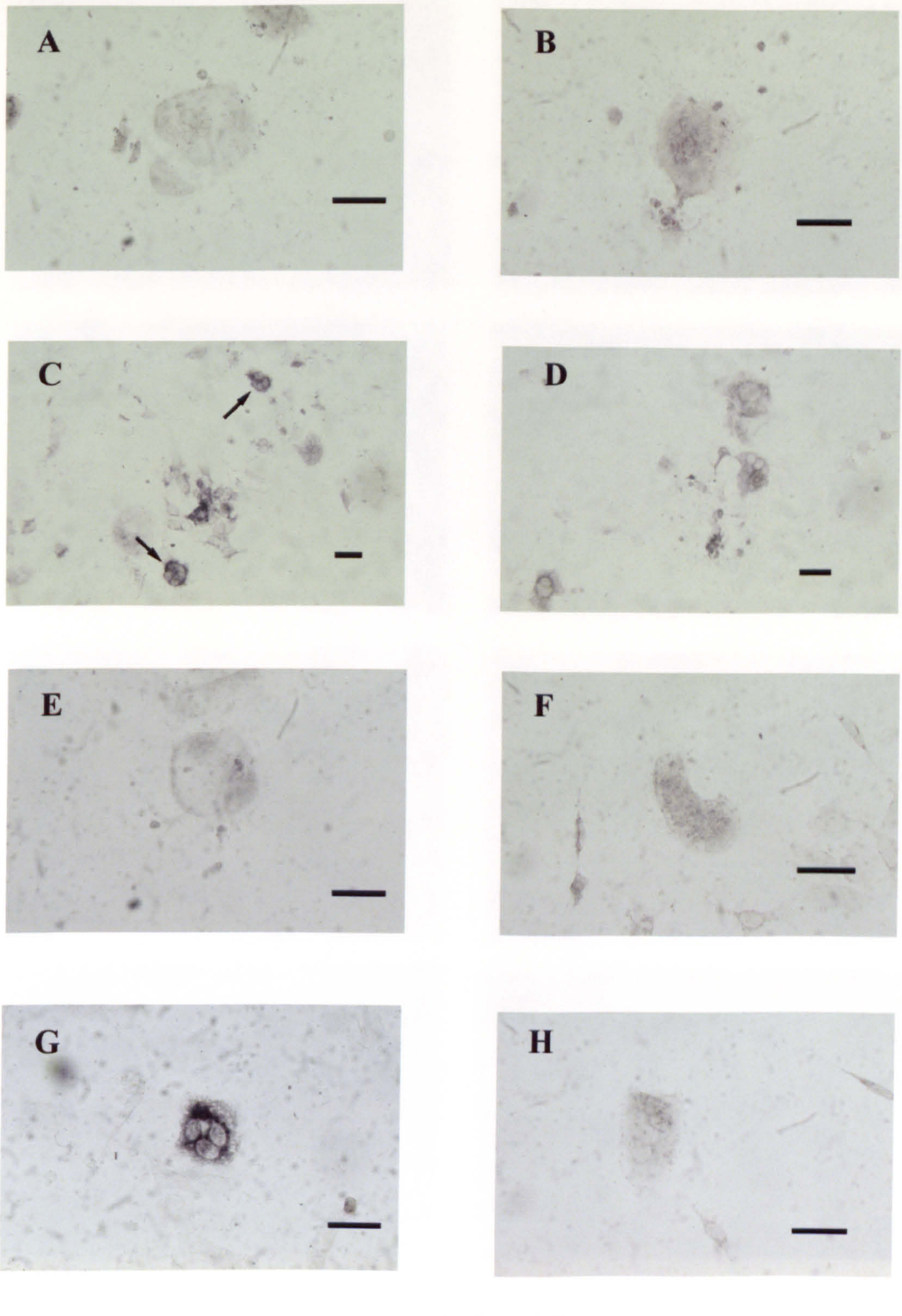


Fig. 5-2. *In situ* hybridization of ADAMs in isolated osteoclasts.
Osteoclasts were prepared as described in Materials and Methods and cultured on rat tail type I collagen coated coverslips. Coverslips were hybridized with **A**, ADAM-9 antisense riboprobe; **B**, ADAM-9 sense riboprobe; **C**, ADAM-12 antisense riboprobe; **D**, ADAM-12 sense riboprobe; **E**, ADAM-19 antisense riboprobe; **F**, ADAM-19 sense riboprobe. **G**, osteoclasts hybridized with MMP-9 antisense and **H**, MMP-9 sense. Bar = 50 μm. Osteoclasts exhibited expression of MMP-9 and also ADAM-12 (arrows).

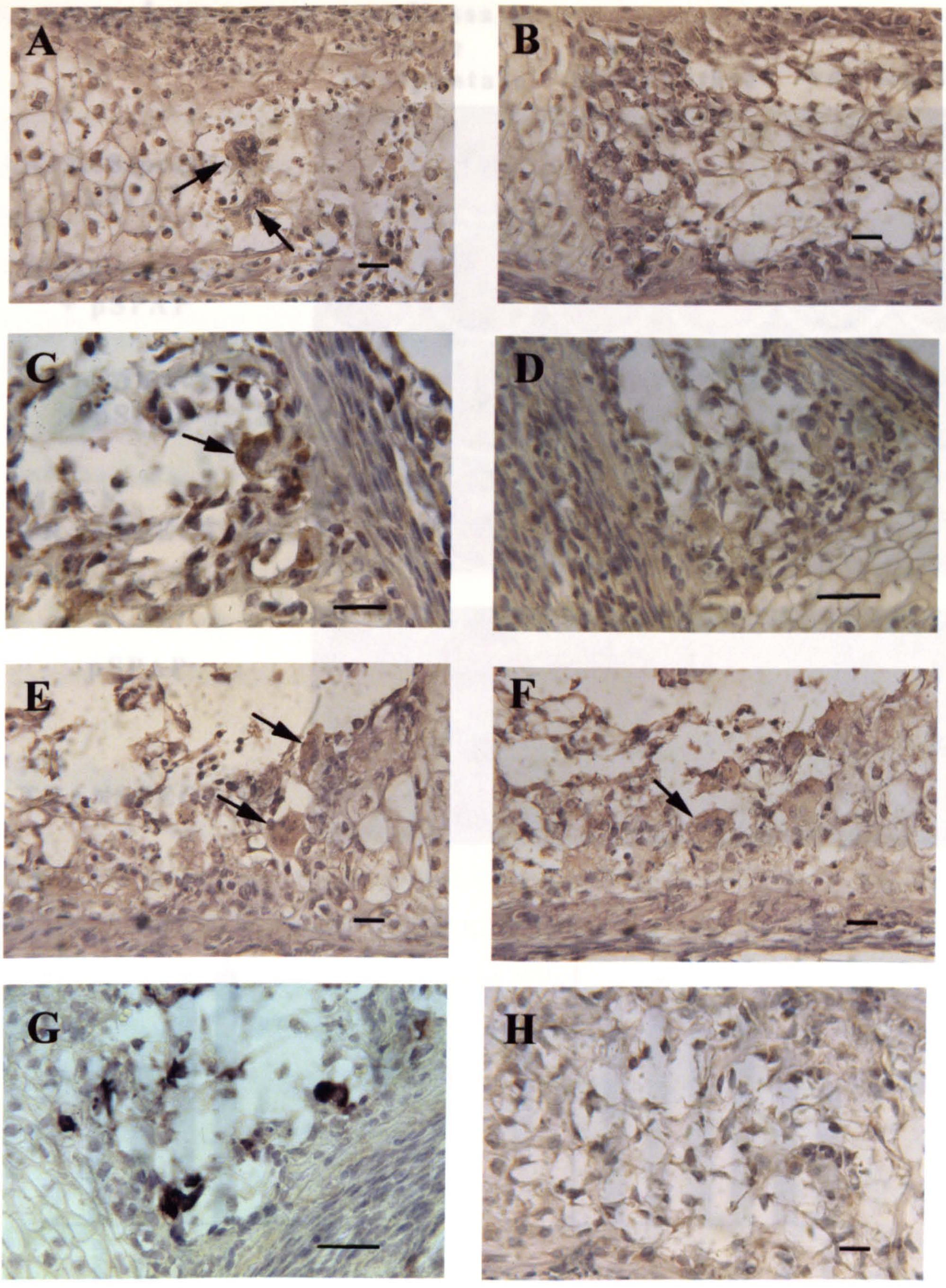


Fig. 5-3. *In situ* hybridization of ADAMs in metatarsals explants.
Metatarsal explants were dissected and cultured as described in section **4.2.2.8**. Sections were hybridized with A, ADAM-9 antisense riboprobe; B, ADAM-9 sense riboprobe; C, ADAM-12 antisense riboprobe; D, ADAM-12 sense riboprobe; E, ADAM-19 antisense riboprobe; F, ADAM-19 sense riboprobe. G, section hybridized with MMP-9 antisense and H, MMP-9 sense. Bar = 50 μm. Arrows show MNCs.

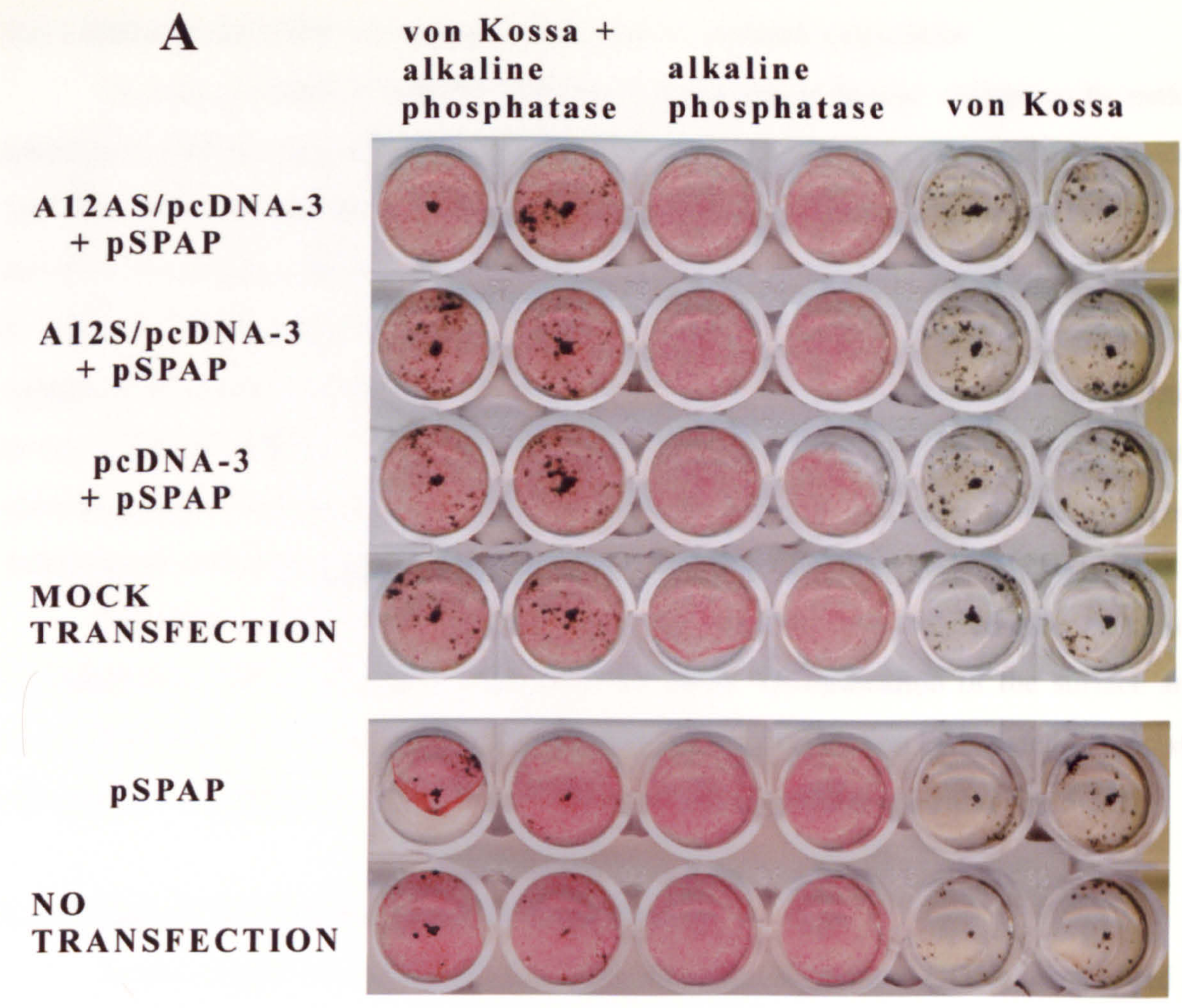


FIG. 5-4. Effect of A12/AS construct on osteoblast differentiation.
Primary mouse osteoblasts were cultured in 24-well plates and transfected as described in Materials and Methods. **A**, After 14 days cultures were stained by the von Kossa method and by alkaline phosphatase. **B,** Culture medium was assayed for SPAP 3 days after transfection. **C**, The presence of mineralized nodules was assessed after 14 days. Results for SPAP levels and % mineralization are expressed as mean ± SEM of 8 wells from 2 separate experiments.

5.3.4 Effect of AS-ODN on lacunar resorption by isolated osteoclasts

In order to establish whether ADAM-12 has a role in lacunar resorption by mature osteoclasts ODNs were added to osteoclasts isolated from the femurs and tibias of 6-day-old CD1 mice. In this study ODNs were used at a concentration of 5 μM based on a previous investigation by Inui *et al.* (1996) on inhibition of lacunar resorption by cathepsin K antisense ODNs. Uptake of ADAM-12 antisense ODNs by isolated osteoclasts was confirmed by addition of a FITC-labelled ODN (5 μM) having the same base composition as the AS-ODN. Figure 5-5A shows an osteoclast, cultured on a type I collagen coated glass coverslip in the presence of FITC-conjugated ODN for 24 h, and exhibiting intense fluorescence in the cytoplasm and nuclei.

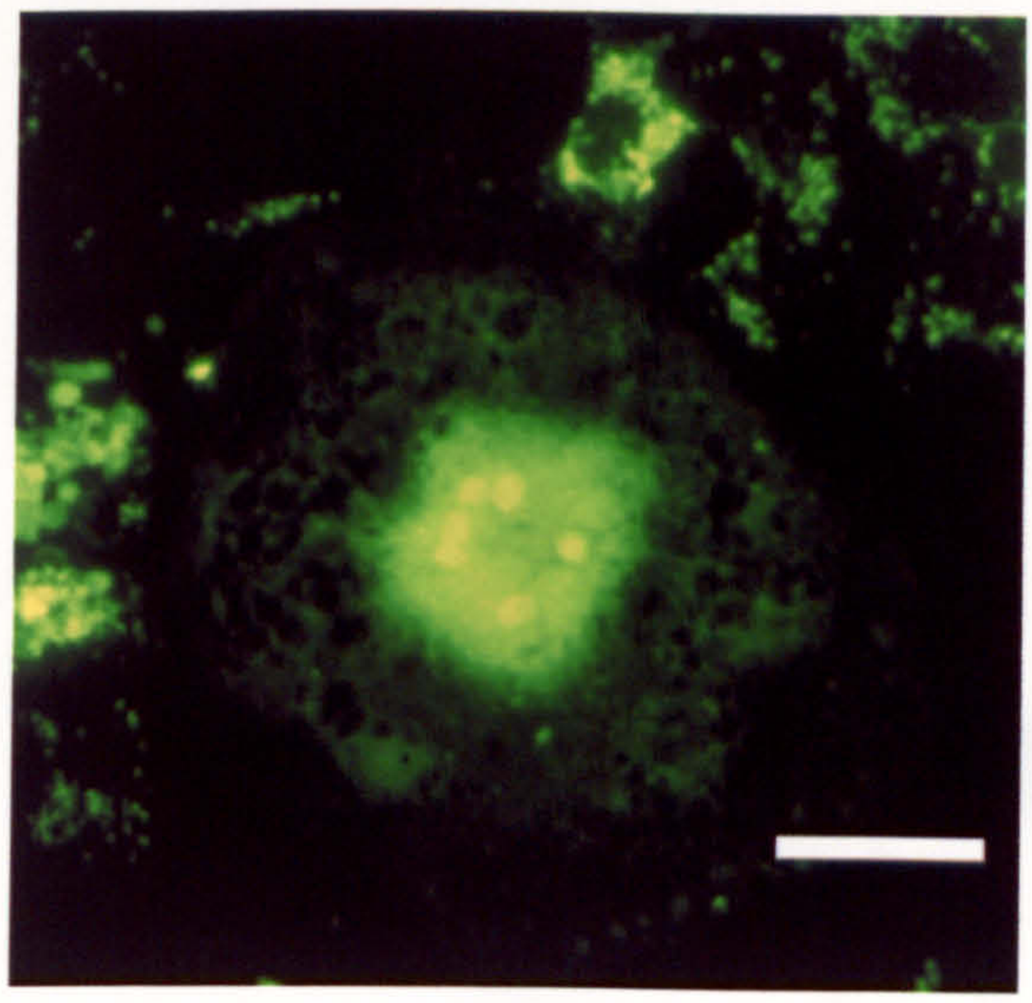
The effect of ADAM-12 AS-ODN on lacunar resorption by isolated osteoclasts was investigated by the pit formation assay on ivory slices. Quantification of the surface area resorbed showed that AS-ODN had little effect on lacunar resorption by isolated osteoclasts with only a 15% decrease compared with the sense and scrambled controls (Fig. 5-5B).

5.3.5 Effect of AS-ODN on osteoclast formation

To investigate the role of ADAM-12 in osteoclast formation, ODNs were added to mouse bone marrow cultures stimulated with 1,25-$(OH)_2D_3$ (10^{-8} M). In the absence of ODN these cultures developed large MNC-osteoclast like cells that were TRAP-positive (78 ± 14 MNCs/well; figure 5-6A). Addition of S-ODN (100 nM) and Scr-ODN (100 nM) had little effect on formation of MNCs in these cultures (68 ± 8; 64 ± 9 MNCs/well respectively). Addition of AS-ODN however resulted in a significant decrease in the number of MNCs formed per well compared to the control, S-ODN, and Scr-ODN.

The effect of AS-ODN formation was also investigated in an alternative assay for osteoclast formation which utilizes spleen cells as the source of haematopoietic precursors stimulated with M-CSF (10 ng/ml) and RANKL (40 ng/ml). Addition of M-CSF and RANKL to this assay obviates the need for osteoblasts in osteoclast formation. In the absence of ODNs or in the presence of S-ODN and Scr-ODN, spleen cell cultures developed large numbers of TRAP-positive MNCs (97 ± 11, 100 ± 10 and 95 ± 9 MNCs/well respectively). Addition of AS-ODN, however, resulted in only a minor inhibition in TRAP-positive MNC formation (80 ± 10 MNCs/well).

A



B

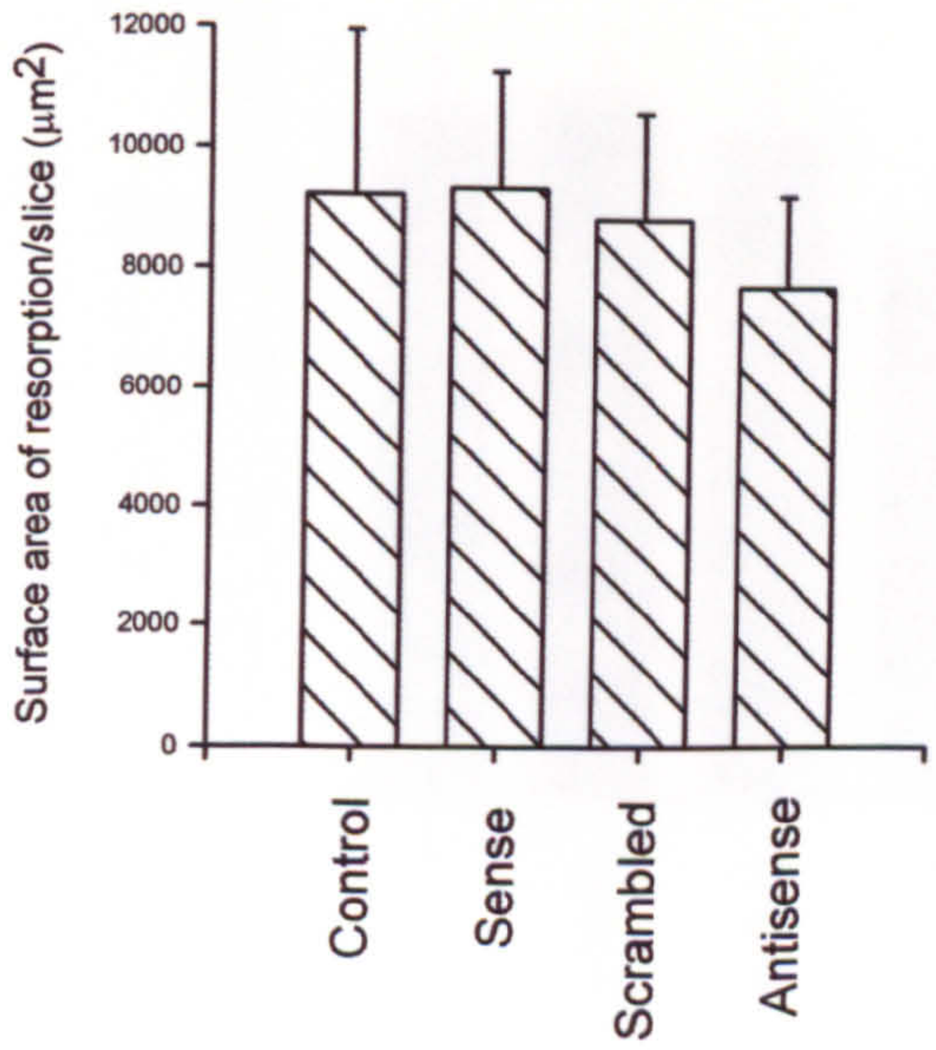


Fig. 5-5. Effects of oligodeoxynucleotides on lacunar resorption by isolated mouse osteoclasts.

Mouse osteoclasts were sedimented onto ivory slices for 25 min and cultured in the presence of ADAM-12 specific oligodeoxynucleotides (5 µM). **A**, Culture in the presence of FITC labelled ODN demonstated uptake of ODN by osteoclasts as revealed by intense fluorescence in the cytoplasm and nuclei. Bar = 50 µm **B**, After incubation for 24 h, resorption was quantified by image analysis. Data are expressed as mean ± SEM of 12 slices from 3 separate experiments. Disruption of ADAM-12 expression had a minimal effect on lacunar resorption.

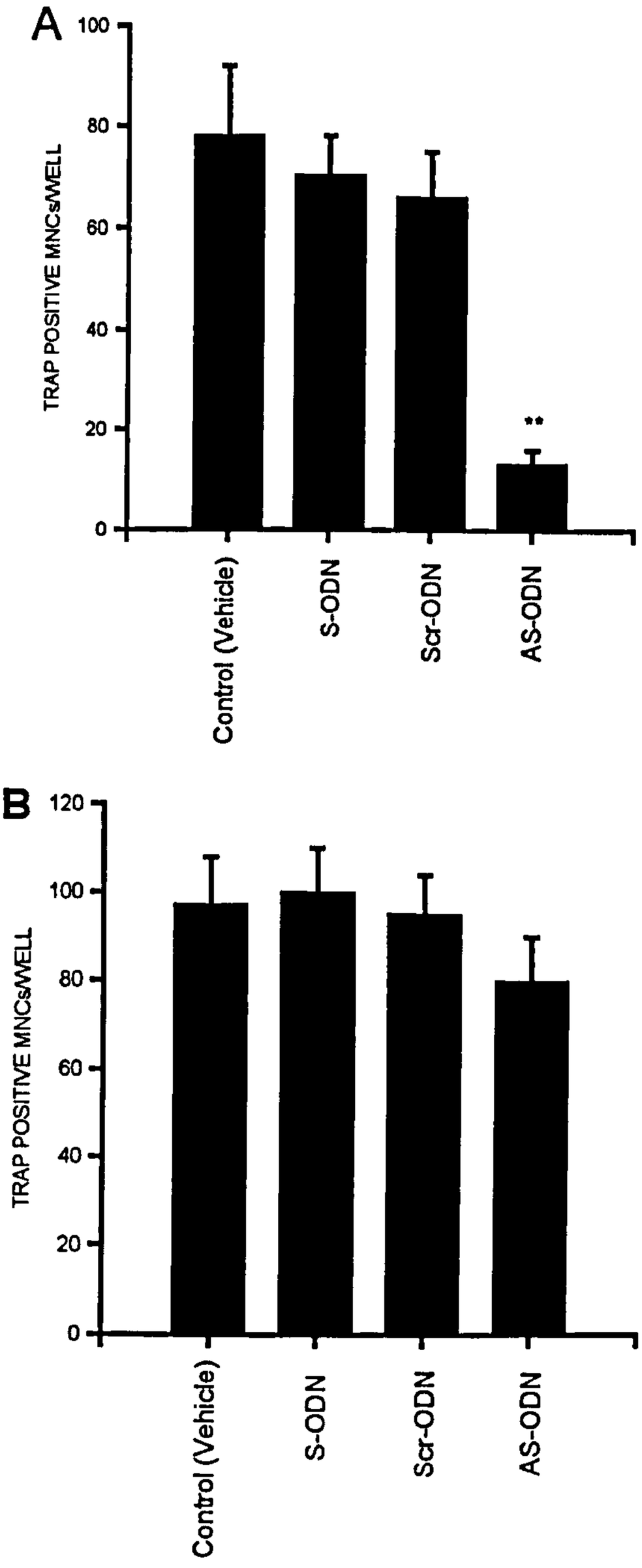


FIG. 5-6. Effects of oligodeoxynucleotides on formation of TRAP-positive MNCs.
A, Mouse bone marrow cells stimulated with 1,25-$(OH)_2D_3$ (10^{-8} M) were cultured in the presence of ODNs (100 nM) for 8 days. In the presence of AS-ODN to ADAM-12 there was an inhibition in TRAP-positive MNC formation compared with bone marrow cells cultured in the presence of S- and Scr-ODNs. Data are expressed as mean ± SEM of 6 wells from 3 experiments. **B**, Mouse spleen cells stimulated with M-CSF (10 ng/ml) and RANKL (40 ng/ml) were cultured in the presence of ODNs (100 nM) for 8 days. AS-ODN to ADAM-12 had only a minimal effect on formation of TRAP-positive MNCs compared with the controls. Data are expressed as mean ± SEM of 6 wells, $^{**}P<0.01$

5.3.6 Effect of A12/cys on osteoclast formation

It has been proposed that the cysteine rich domain of ADAM-12 may be involved in cellular fusion based on the existence of a sequence similar to the potential fusion peptide of rubella virus (Yagami-Hiromasa *et al.*, 1995). If the cysteine rich domain is involved in an aspect of osteoclast formation then addition of recombinant A12/cys to osteoclast formation assays may compete with endogenous ADAM-12 for binding to its target thereby, acting as a mimetic in a similar way to RGD containing peptides. A12/cys was expressed in this study as a fusion protein with GST in *E.coli*. The fusion protein was purified from bacterial extracts on Glutathione-sepharose and buffer exchanged against 10 mM Tris pH 7.4. Analysis of the fusion protein by SDS-PAGE showed that the fusion protein migrated at an expected molecular weight of approximately 40 kDa (Fig. 5-6A). To investigate whether the cysteine rich domain of ADAM-12 is involved in fusion of osteoclast precursors, M-CSF dependent bone marrow cells (MDBM) stimulated with M-CSF (10 ng/ml) and RANKL (40 ng/ml) were cultured in the presence recombinant A12/cys (10^{-8} M) or recombinant GST (10^{-8} M) as a control. This assay was chosen due to its greater potential to form large numbers of TRAP-positive MNCs with characteristics of osteoclasts (Fig. 5-6B and C). Addition of recombinant GST to MDBM cell cultures had no effect on formation of TRAP-positive MNCs (Fig. 5-7B) or on resorptive activity of osteoclasts generated in this assay (Fig. 5-7C). However, addition of A12/cys resulted in a significant inhibition of both osteoclast formation (37 ± 10 MNCs/well) and osteoclast resorptive activity compared with the GST control and the control without added compound.

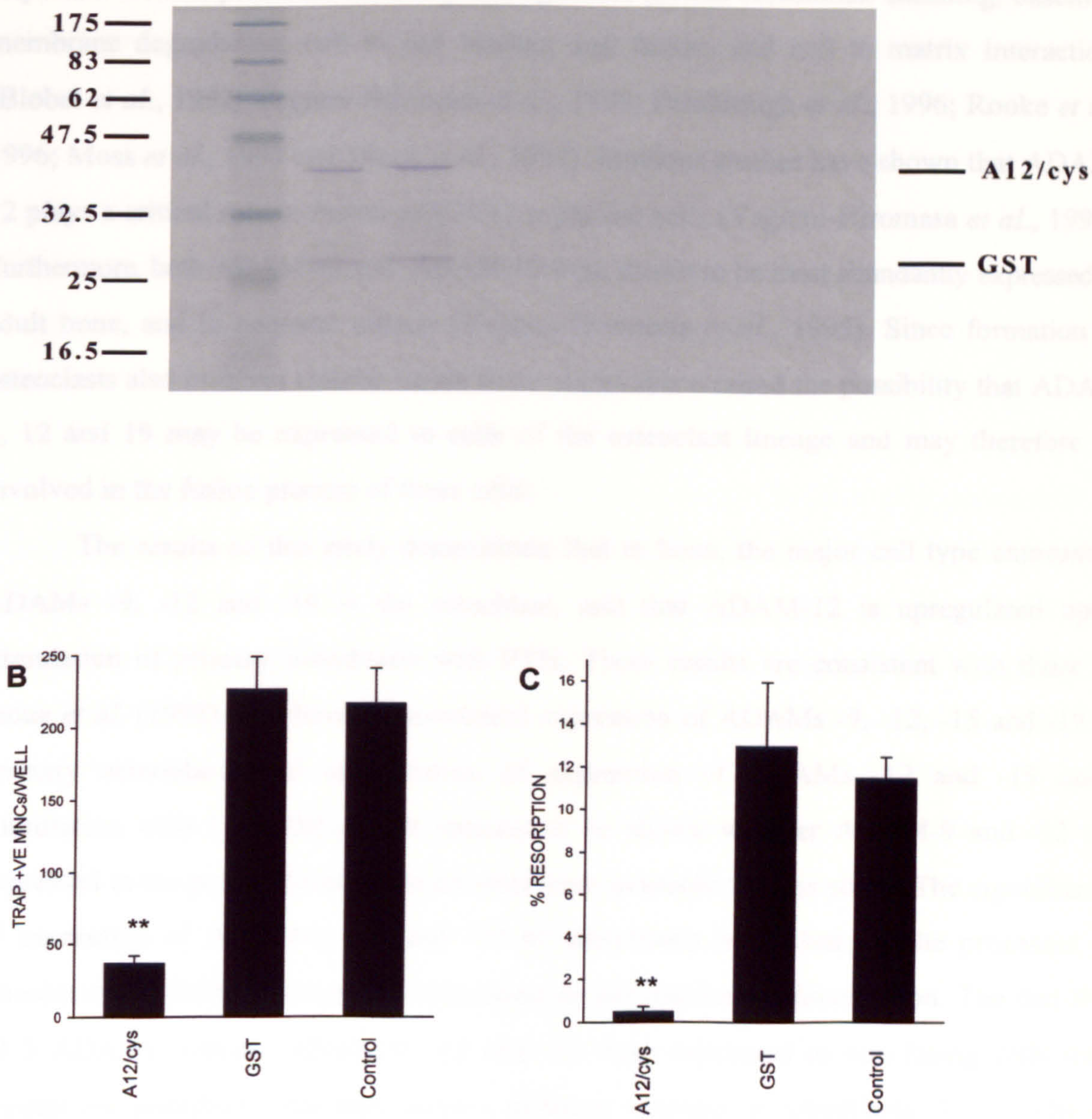


FIG. 5-7. Effects of A12/cys on osteoclast formation.
A12/cys was purified on GST sepharose and buffer exchanged with 10 mM Tris.Cl pH 7.4 **A**, SDS-PAGE of recombinant A12/cys, run under reducing conditions, used to investigate osteoclast formation. Lane 1, molecular weight markers; lanes 2-3 cysteine rich domain GST fusion protein of mouse ADAM-12. M-CSF dependent bone marrow cells were cultured in 48-well plates and stimulated with M-CSF (10 ng/ml) and RANKL (40 ng/ml). Recombinant A12/cys was added at a concentration of 10^{-8} M. As a control recombinant GST was added to some wells at a concentration of 10^{-8} M. Control wells received vehicle (5 µl 10 mM Tris.Cl pH 7.4). Addition of A12/cys significantly reduced TRAP-positive MNC formation **B** and resorption **C**. The results are the mean ± SEM of 24 separate cultures from 3 separate experiments. The inhibitory effect was statistically significant, $^{**}P<0.01$ compared with cultures in the presence of GST and control in the absence of any compound.

5.4 Discussion

Members of the ADAM family of matrixins have been implicated in a variety of important cellular processes including neurogenesis, protein ectodermal shedding, basement membrane degradation, cell to cell binding and fusion, and cell to matrix interactions (Blobel *et al.*, 1992; Yagami-Hiromasa *et al.*, 1995; Fambrough *et al.*, 1996; Rooke *et al.*, 1996; Moss *et al.*, 1997 and Black *et al.*, 1997). Previous studies have shown that ADAM-12 plays a critical role in fusion of C2C12 myoblast cells (Yagami-Hiromasa *et al.*, 1995). Furthermore, both ADAM-12 and ADAM-19 were shown to be most abundantly expressed in adult bone, and in neonatal muscle (Yagami-Hiromasa *et al.*, 1995). Since formation of osteoclasts also involves cellular fusion these observations raised the possibility that ADAM 9, 12 and 19 may be expressed in cells of the osteoclast lineage and may therefore be involved in the fusion process of these cells.

The results of this study demonstrate that in bone, the major cell type expressing ADAMs -9, -12 and -19 is the osteoblast, and that ADAM-12 is upregulated upon stimulation of primary osteoblasts with PTH. These results are consistent with those of Inoue *et al.* (1998) who have demonstrated expression of ADAMs -9, -12, -15 and -19 in primary osteoblasts and upregulation of expression of ADAMs -12 and -19 upon stimulation with 1,25-$(OH)_2D_3$. It remains to be shown whether ADAM-9 and -12 are expressed at the protein level: no antibodies were available for this study. The significance of expression of ADAM-9, -12 and -19 by osteoblasts in unclear, as the processes of osteoblast cell differentiation and maturation do not require multinucleation. The fact that all 3 ADAMs, namely ADAM-9, -12 and -19 were expressed in non-fusing cells may suggest the possibility that they serve a different function in osteoblasts. The results of Inoue *et al.* (1998) show that ADAM-12 and ADAM-19 are regulated in a differentiation-associated manner in the mouse osteoblastic cell line MC3T3E1 implying a role in osteoblast differentiation. It has been shown IGFBP-5 levels in culture supernatants of differentiating MC3T3-E1 cells decrease during differentiation which is associated with a 97 kDa proteinase which was not an MMP (Thraikill *et al.*, 1995). It has subsequently been shown that a soluble form of human ADAM-12 (ADAM 12-S; Loechel *et al.*, 1998) can bind and cleave IGFBP-3 and -5 (Shi *et al.*, 2000; Loechel *et al.*, 2000). In this study transfection of primary mouse osteoblasts with antisense constructs against ADAM-12 had no effect on differentiation of osteoblasts and formation of bone nodules. It may be that the disruption of expression of ADAM-12 in this assay is compensated for by ADAM-19 or

another member of the ADAM family. It remains to be seen whether disruption of ADAM-19 alone or in combination with ADAM-12 has any effect on osteoblast differentiation.

A more likely function for ADAMs -9, -12, -19 and -15 in osteoblasts is to promote cell-cell and cell-matrix interactions. Interestingly, ADAM-9 has recently been shown to function as an adhesion protein by binding the $\alpha_v\beta_5$ integrin in a non RGD dependent fashion (Zhou *et al.*, 2001). Similarly ADAM-15 has been shown to bind to the $\alpha_v\beta_3$ integrin (Nath *et al.*, 1999). The $\alpha_v\beta_3$ integrin has been shown to be expressed in osteoclasts (Nesbitt et al., 1993) and thus ADAM-9 and -15 may promote osteoblast-osteoclast interactions. ADAM-12 has been shown to interact with the $\alpha_9\beta_1$ integrin in a non RGD dependent fashion via its disintegrin domain (Eto *et al.*, 2000) although expression of $\alpha_9\beta_1$ integrin in bone cells has not been shown.

The demonstration of ADAM-12 expression in isolated osteoclasts suggests that this proteinase may be involved in an aspect of the bone resorption cascade. Previous studies have shown that MMPs and cysteine proteinases are key proteolytic enzymes in the degradation of the organic matrix of bone (Hill *et al.*, 1994a; 1994b). In this study addition of ADAM-12 AS-ODN to cultures of primary isolated osteoclasts produced only a 15% decrease in lacunar resorption by isolated osteoclasts. This suggests that ADAM-12 is not a key player in the degradation of the organic matrix. By comparison a similar study by Inui *et al.* (1996) demonstrated a 50% reduction in lacunar resorption upon disruption of cathepsin K by ODNs. The metalloproteinase domain of ADAM-12 has been shown to be catalytically active by using the trapping mechanism of α_2-macroglobulin to assay for protease activity (Loechel *et al.*, 1998). At present no physiological substrate for membrane-bound ADAM-12 has yet been identified although ADAM 12-S can cleave IGFBP-5 and this interaction has been shown to be inhibited by TIMP-3 (Loechel *et al.*, 2000). Only ADAM-10 has been shown to have proteolytic activity against an ECM protein being able to degrade type IV collagen (Millichip *et al.*, 1998) as well as a non ECM protein, myelin basic protein (Howard *et al.*, 1996). It seems that the metalloproteinase domain of catalytically active ADAM proteinases are predominantly involved in proteolytic shedding of membrane bound cytokines and growth factors (Schlondorff and Blobel, 1999). Interestingly, recent studies have shown that ADAM-9 is involved in the processing of heparin binding EGF-like growth factor (Izumi *et al.*, 1998) and ADAM-19 is involved in the processing of neuregulin

(Shirakabe *et al.*, 2001). It seems likely that ADAM-12 may be involved in the protein ectodomain shedding.

The findings that ADAM-12 is expressed in osteoclasts of cultured 17-day-old fetal mouse metatarsal suggest that ADAM-12 may play a role in migration of osteoclast precusors and formation of multinucleated osteoclasts. These results are consistent with previous studies by Abe *et al.* (1999) demonstrating expression of ADAM-12 in osteoclasts generated in bone marrow cultures. Disruption of ADAM-12 expression in this study using antisense ODNs led to an inhibition of osteoclast formation in the bone marrow assay. These results are consistent with previous findings by Abe *et al.* (1999). However, antisense ODNs had little effect on osteoclast formation in cultures of spleen cells treated with RANKL and M-CSF. Since the spleen cell/RANKL/M-CSF assay for osteoclast generation has no mesenchymal/osteoblastic cells present, these results suggest a role for ADAM-12 in mediating a cell-cell interaction between osteoblasts and osteoclast precursors which is an essential pre-requisite for osteoclast formation. Another possibility is that treatment of spleen cells with RANKL and M-CSF may alter the expression of ADAM family members such that disruption of ADAM-12 is compensated for by other family members. Interestingly, ADAM-8 has also been shown to be expressed in haemopoietic cells and demonstrated to be a novel osteoclast stimulating factor (Choi *et al.*, 2001). Previous studies have suggested that ADAM-12 may be directly involved in the process of cellular fusion (Yagami-Hiromassa *et al.*, 1995) consistent with the presence of a potential hydrophobic fusion peptide sequence within the cysteine rich domain. Results from this study on the effect of addition of recombinant A12/cys to cultures of MDBM cells suggest that the cysteine rich domain may have a pivotal role in osteoclast formation. Such a role may involve a cell-cell interaction between osteoclast precursors which is a prerequisite for fusion. Recent studies using recombinant human ADAM-12 cysteine rich domain have shown that it supports cell adhesion through syndecans (Iba *et al.*, 2000).

6. Effects of Serine Proteinase inhibitors on Bone Resorption *In Vitro*

6.1 INTRODUCTION

Bone resorption involves the removal of both the mineral and organic constituents of bone matrix. Osteoclasts are the cells principally responsible for this process which occurs in the subosteoclastic resorption zone (SORZ), a specialized extracellular compartment bounded by the ruffled border of the cell and the mineralized bone matrix (Baron, 1989). Osteoclasts acidify the SORZ leading to dissolution of mineral (Blair *et al.*, 1989) while the organic matrix is believed to be degraded by lysosomal cysteine proteinases, matrix metalloproteinases and also serine proteinases.

The plasminogen activators (PAs), uPA and tPA, are serine proteinases (SPs) that catalyze the conversion of the proenzyme plasminogen into a broad spectrum serine-protease, plasmin. The plasmin thus generated can either directly or indirectly, via activation of latent MMPs, promote the degradation of all components of the extracellular matrix *in vitro* (Mignatti and Rifkin, 1993; Murphy *et al.*, 1992). This latter activity may be relevant to the physiological process involved in osteoclast migration. With regard to a possible involvement of the SPs in bone turnover, plasminogen has been shown to be present in extracellular matrices (Knudsen *et al.*, 1986). Furthermore osteoblasts produce PAs in response to agents that promote bone resorption (Fukumoto *et al.*, 1992; Allan and Martin, 1995) and most recently it has been suggested that SPs are involved in the degradation of non-collagenous proteins of bone (Daci *et al.*, 1999). Although these results support the notion that the PA/plasmin system might be involved in bone resorption results from other studies suggest a limited role (Leloup *et al.*, 1994; 1996). Since plasminogen is the most abundant and best defined substrate for PAs and that its activation by PAs results in the generation of plasmin (Vassalli *et al.*, 1991), the importance of plasmin activity to the normal sequence of events that leads to osteoclastic bone resorption was investigated in this study.

The aim of this study was to assess the contribution of the PA/plasmin system to the different aspects of the bone resorption cascade using selective inhibitors of SPs in combination with a variety of *in vitro* models that are specific for the various aspects of the

resorption process. Furthermore an assessment was made of the expression of the SPs and their natural inhibitors in these model systems by RT-PCR and *in situ* hybridization.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Cell culture reagents and the SP inhibitors, aprotinin and α2-antiplasmin were purchased from Sigma Chemical Co. (Poole, Dorset, UK). The specific gelatinase inhibitor, CT1399 was a gift from Dr A Docherty, CellTech, UK. $^{45}CaCl_2$, ^{14}C and ^{3}H-amino acid mixture were purchased from Amersham International (Aylesbury, United Kingdom). Enzymes and reagents for RT-PCR were purchased as a kit from Perkin Elmer Biosystems (California, USA). The cysteine proteinase inhibitor Ep453 was a generous gift from Dr. M. Murata (Research Centre, Taisho Pharmaceuticals, Saitama, Japan) and inhibits the activities of cathepsins B, L, S and K.

6.2.2 Methods

6.2.2.1 Neonatal calvarial assay

Bone reorption was assessed by analysing $^{45}Ca^{2+}$ release from neonatal mouse calvarial bones as described in section **4.2.2.1**. The bones were precultured for 24 h in CMRL-1066 medium (2 ml) containing 5% acid-treated rabbit serum and indomethacin (1μM). Bones were subsequently cultured in pairs in fresh CMRL-1066 medium (2 ml) containing 5% FBS and stimulated with either PTH or 1,25-$(OH)_2D_3$ (10^{-8} M) for upto 4 days with media change every 2 days in the presence or absence of SP inhibitor. Release of $^{45}Ca^{2+}$ was determined as described in section **4.2.2.1**.

6.2.2.2 Isolated osteoclast assay

The isolated osteoclast assay was carried out as described in section **4.2.2.6**. Ivory slices were incubated for 24 h in 500 μl α-MEM supplemented with 5% FBS, 2.0g/liter $NaHCO_3$, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin. Cultures were stimulated with IL-1α (10^{-10} M) in the presence or absence of SP inhibitor, the MMP inhibitor CT1399 or Ep453.

6.2.2.3 Osteoclast formation assay

Osteoclasts were generated in a coculture system consisting of bone marrow of 5- to 6-week-old mice and primary osteoblasts. Primary mouse osteoblasts were plated at a density of 2 x 10^4 cells/well of a 24-well plate in α-MEM containing 10% FBS and cultured overnight. Bone marrow was isolated as described in section **4.2.2.7** and added at a cell density of 1 x 10^6 cells per well in 0.5 ml α-MEM containing 10% FBS and 10^{-8} M 1,25-$(OH)_2D_3$. Osteoclast formation was assessed by counting the number of TRAP-positive multinucleated cells with over two nuclei after 8 days in culture.

6.2.2.4 Fetal metatarsal long bone assay

Osteoclast migration was assessed using 17-day-old fetal metatarsals prepared as described in section **4.2.2.8**. The metatarsal explants were cultured for 3 days and then fixed in 4% paraformaldehyde overnight at 4°C. Specimens were then washed in PBS, decalcified in 5% EDTA overnight, dehydrated through a graded series of ethanol and embedded in paraffin. Sections of 5 μm were cut, transferred to sialinised glass slides (Sigma) and dried at 50°C overnight.

6.2.2.5 Histomorphometry

The number of TRAP-positive cells and their nuclei were determined in 10 evenly spaced longitudinal sections per long bone rudiment. According to their location they were scored as (a) lying in the developing marrow cavity, that is the area of resorbing calcified cartilage surrounded by the thin bone collar; or (b) in the periosteum-perichondrium, that is, the soft tissue around the bone rudiment. The few cells lying within the (thin) bone collar were equally divided over the two compartments.

6.2.2.6 Preparation of osteoblasts from neonatal mouse calvariae

Calvarial osteoblasts were prepared and characterized as previously described in section **2.2.2.1**

6.2.2.7 Preparation of collagen films and acid-treated serum

Radiolabelled collagen films were prepared by the method described previously in section **4.2.2.3**. Acid-treated serum was prepared as described in section **4.2.2.4**

6.2.2.8 Culture of osteoblasts on collagen films

Osteoblasts (1 x 10^5/well) were settled onto collagen films in 1 ml of DMEM plus 10% (v/v) FCS, incubated for 16 h at 37°C and washed with serum-free DMEM. Cells were then cultured in DMEM (1 ml) supplemented with 5% (v/v) acid-treated rabbit serum as described above. Either 1,25-$(OH)_2D_3$ (10^{-8} M) alone or 1,25-$(OH)_2D_3$ plus either aprotinin or CT1399 was then added to the wells and the cultures maintained at 37°C for 48 h. The basal release of ^{14}C by unstimulated osteoblasts was subtracted from the 1,25-$(OH)_2D_3$-stimulated release in the presence and absence of inhibitors to give the corrected values for stimulated lysis. At the end of the culture period the media were centrifuged (15 min, 1200x*g*) to remove any collagen fibrils and radioactivity released during collagen degradation quantified by liquid scintillation counting. Residual collagen was digested with bacterial collagenase (50 μg/ml) and assayed for radioactivity. Collagenolysis was expressed as radioactivity released from the films as a percentage of the total ± SEM.

6.2.2.9 Formation of ^3H-labelled extracellular bone matrix

The murine calvaria-derived cell line, MC3T3-E1, is a well characterized osteoblast culture system providing a suitable model of osteogenesis analogous to *in vivo* bone formation (Sudo *et al.*, 1983). When cultured in medium containing ascorbic acid, these cells have been shown to express phenotypic markers characteristic of mature osteoblasts including alkaline phosphatase, osteocalcin, osteopontin, type I collagen synthesis and formation of an extracellular collagenous matrix (Kurihara *et al.*, 1986; Boyan *et al.*, 1989; Quarles *et al.*, 1992).

Extracellular bone-like matrix was produced as described by Ronday *et al.* (1997). MC3T3-E1 cells were plated at a density of 1 x 10^4 cells/well on collagen-coated 24-well plates (Becton Dickinson, MA, USA) and cultured in α–MEM supplemented with 10% FBS, 50 μg/ml ascorbic acid. After 4-5 days, when the cultures had reached confluence and the formation of an extracellular matrix had started, fresh medium was added containing 1 μCi/ml ^3H amino acid mixture (Amersham International, Aylesbury, UK) to create a non-mineralized radiolabelled extracellular bone matrix. The radiolabelled medium was changed every 72 h. After 14 days, cells were lysed with 0.5 ml/well of Triton X-100 (0.5% v/v in PBS). The cytoskeleton was removed by 25 mM NH_4OH treatment and 1mM

phenylmethylsulphonyl fluoride (0.5 ml/well) treatment was used to block proteinase activity. Matrices were washed with H_2O and 75% (v/v) ethanol to remove unincorporated ^{3}H-radiolabelled amino acids, dried and stored at -20°C.

6.2.2.10 Bone matrix degradation assay

To assay for matrix degradation, osteoblasts were plated onto the matrices at a density of 1 x 10^5 cells/well in culture medium with and without specific proteinase inhibitors. All culture media were supplemented with 10% acid-treated FBS to maintain cell viability. Aprotinin or CT-1399 each at a concentration of 10^{-5} M were added to different wells. After 72 h incubation, the media were removed and the extent of degraded ^{3}H-radiolabelled matrix released into the medium was determined by liquid scintillation counting. The remaining matrix was degraded with 0.25% (w/v) trypsin, 0.1% (w/v) collagenase in PBS for 1 h at 37°C, and the amount of radioactivity in the matrix similarly assessed. Matrix degradation was expressed as ^{3}H released in the medium as a percentage of the total amount of ^{3}H released by the cells during the 72 h culture period, plus that solubilized from the remaining matrix by trypsin/collagenase treatment.

6.2.2.11 Extraction of total RNA

Total RNA was prepared as described in section **5.2.2.1**. The RNA pellet was solubilised in diethyl pyrocarbonate (DEPC)-treated water, and the concentration of the RNA was determined spectrophotometrically (Ultrospec III, Pharmacia, St. Albans, UK).

6.2.2.12 RT-PCR procedure

Synthetic oligonucleotide sequences specific for tPA, uPA, uPAR, PAI-1, PAI-2 and PN-1 were synthesized by Life Technologies Ltd. (Paisley, UK) using previously published sequence data (Yang *et al.*, 1997) and shown in Table 1. The housekeeping gene GAPDH was used as a positive control for the RT-PCR methodology.

6.2.2.13 RT-PCR and preparation of probes

RT-PCR was performed as described in section **5.2.2.2** under the same conditions.

RT-PCR products were analysed by gel electrophoresis on a 2% agarose gel. The DNA fragment corresponding to tPA was excised from the gel and cloned into pGEM-T as described in section **5.2.2.3**.

The probe for uPA was a generous gift from Dr. J.D. Vassalli (University of Geneva) and consisted of a 660 base pair fragment cloned into the PstI/HindIII site of plasmids pSP64. The plasmids were transformed to competent JM109 *E. coli* and recombinant clones selected for on LB medium containing ampicillin (100 μg/ml). *E. coli* were then cultured in 250 ml of LB broth containing ampicillin (100 μg/ml) and plasmid DNA extracted using a method based on Qiagen Maxiprep DNA purification (Qiagen). Plasmids were linearized with BamH1 for synthesis of Digoxigenin-11-UTP riboprobes.

In situ hybridization was performed as described in section **5.2.2.6**.

Table 6-1. Oligonucleotide RT-PCR primer sequences for mouse PAs.

Gene	Primer Sequence		Expected Product Length (bp)
tPA	F	5'-GACGATACTTATGACAACGAC-3'	255
	R	5'-TATTAAACAGATGCTGTGAGG-3'	
uPA	F	5'-CGAATACTACAGGGAAGAC-3'	219
	R	5'-GACATTTTCAGGTTCTTTGG-3'	
uPAR1	F	5'-ATTGCCTCTCTGCTCCTGAC-3'	331
	R	5'-GAGACCCAACCTTATTCACTG-3'	
PAI-1	F	5'-ATCCTGCCTAAGTTCTCTCTG-3'	290
	R	5'-ATTGTCTCTGTCGGGTTGTG-3'	
PAI-2	F	5'-CAAAGCTGAACATTGGATAC-3'	205
	R	5'-ACCACAACATCATCTTCATC-3'	
PN-1	F	5'-GCGATATAATGTAAACGGAG-3'	225
	R	5'-CAAAAATTGATGGACTCAGAG-3'	
GAPDH	F	5'-CCACGAGAAATATGACAAC-3'	222
	R	5'-GATGCAGGGATGATGTTC-3'	

6.2.2.14 Statistical Analysis

Data are expressed as the mean ± SEM. Differences between control and treatment groups were determined by the Mann-Whitney U test. *, **, *** significantly different from control at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

6.3 RESULTS

6.3.1 Calvarial bone resorption

To determine the role of SPs in bone resorption, a neonatal calvarial assay was used. This assay simultaneously screens for activities influencing various aspects of the bone

resorption process including osteoclast formation and activity. A comparison of the effects of the SP inhibitors aprotinin and α_2-antiplasmin on $^{45}Ca^{2+}$ release from prelabelled calvarial explants was initially carried out. As shown in figure 6-1, each SP inhibitor dose-dependently inhibited $^{45}Ca^{2+}$ release, in the range 10^{-10} to 10^{-5} M. During the 2 days of culture aprotinin and α_2-antiplasmin produced approximately a 50% inhibition of $^{45}Ca^{2+}$ release at a concentration of 10^{-5} M. In contrast the MMP inhibitor CT1399 produced a more complete inhibition of $^{45}Ca^{2+}$ release (76 ± 7%) at a concentration of 10^{-5} M.

To determine whether the inhibitory effect was reversible a recovery experiment was carried out. Calvarial bones were treated with PTH (10^{-9} M) and either aprotinin (10^{-5} M) or α_2-antiplasmin (10^{-5} M) for the first 48 h and then cultured in the presence of PTH only for 48-96 h. The inhibitory effects of the compounds seen during the initial culture period were subsequently lost (Table 6-2). PTH-stimulated release of $^{45}Ca^{2+}$ returned to levels observed without addition of inhibitors during the 0-48 h culture period.

6.3.2 Osteoclast pit formation on ivory slices

The direct effect of the inhibitors on osteoclast function was investigated in an isolated osteoclast pit assay. Both the number of pits formed and their plan area were quantitated. Figure 6-2 shows that aprotinin did not prevent osteoclast resorption on ivory slices, producing only a 2% inhibition in pit number and 5% inhibition in surface area resorbed. In contrast a CP inhibitor, Ep453 (10^{-5} M), produced significant inhibition of osteoclast activity of approximately 80% for both number of pits and the surface area resorbed. Similarly, the MMP inhibitor CT1399 produced a significant decrease in osteoclast activity of approximately 45% for pit number and surface area resorbed.

6.3.3 Osteoclast formation *in vitro*

A coculture system, comprising murine bone marrow cells and primary osteoblasts stimulated with 1,25-$(OH)_2D_3$ was used to assess the effects of the SPs on osteoclast formation. Maximal numbers of osteoclasts, identified as TRAP +ve MNC were formed by 1,25-$(OH)_2D_3$ at a concentration of 10^{-8} M. The addition of either aprotinin (10^{-5} M), or α2-antiplasmin to the cultures did not effect the number of TRAP +ve MNC formed (Table 6-3).

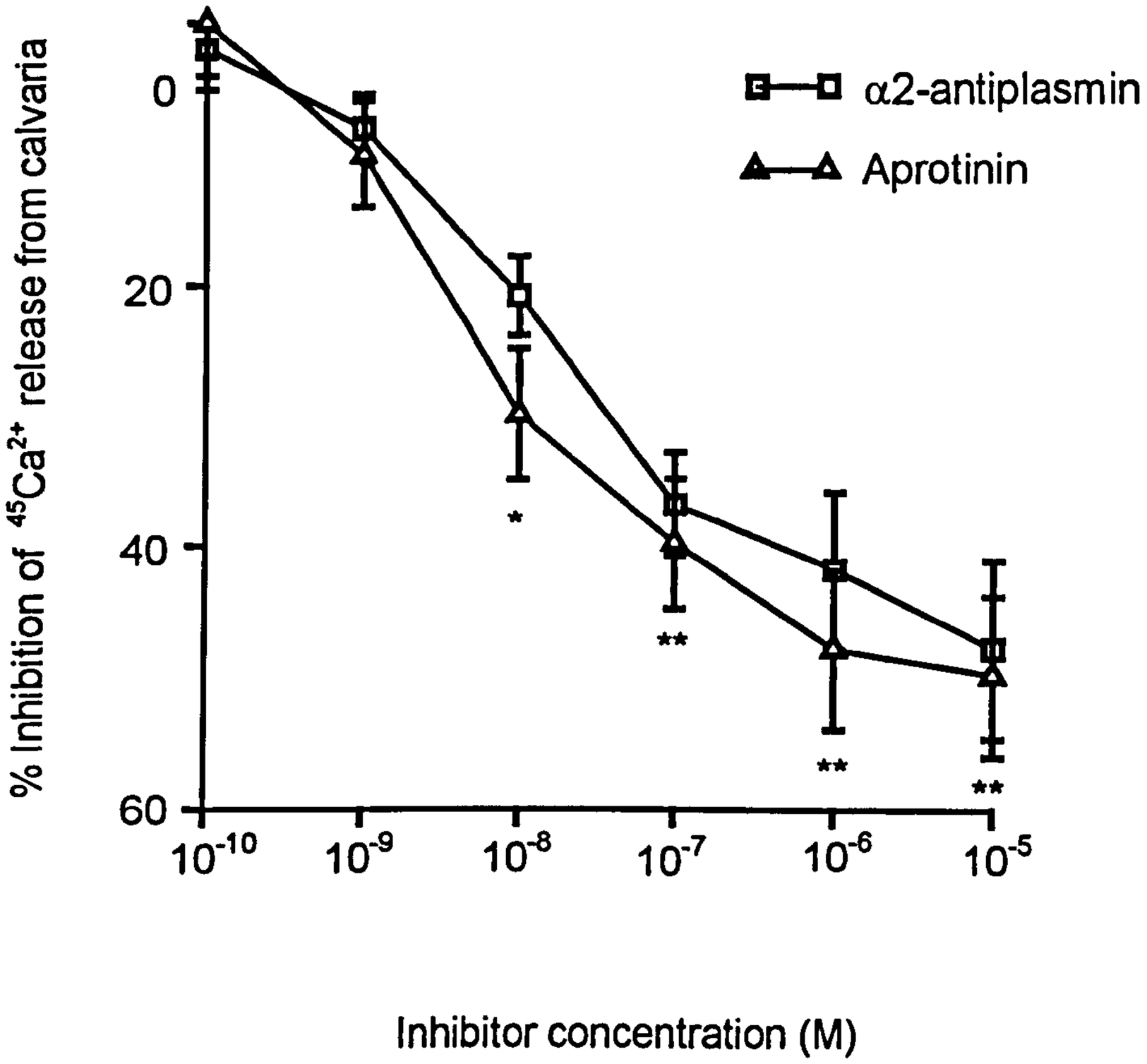


Fig. 6-1. Effects of SP inhibitors on PTH-stimulated release of $^{45}Ca^{2+}$ from calvarial bones after a 48 h incubation period.

The results are expressed as percentage inhibition of PTH-stimulated $^{45}Ca^{2+}$ release which was arbitrarily set to 100%. Each value is the mean ± SEM of 5 pairs of bones. The inhibitory effects of aprotinin (10^{-8}-10^{-5} M) and α_2-antiplasmin (10^{-8}-10^{-5} M) were statistically significant (*$p<0.05$, **$p<0.01$). The percentage release of $^{45}Ca^{2+}$ from PTH stimulated bones was 25.6 ± 4.3 (aprotinin) and 23.6 ± 3.6 (α_2-antiplasmin). Both inhibitors dose-dependently inhibited release of $^{45}Ca^{2+}$.

Table 6-2 Recovery from the inhibitory effects of aprotinin and α2-antiplasmin on the PTH stimulated release of $^{45}Ca^{2+}$ from mouse calvarial bones in culture.

Treatment		Cell-mediated $^{45}Ca^{2+}$ release (%)	
0-48 h	49-96 h	0-48 h	49-96 h
PTH	PTH	17.2 ± 2.1	13.3 ± 2.6
PTH + aprotinin	PTH	9.1 ± 1.5*	15.2 ± 2.1
PTH + α2-antiplasmin	PTH	8.3 ± 2.3*	12.6 ± 1.6

Values are means ± SEM for five calvarial bones prelabelled with 0.37 Mbq $^{45}Ca^{2+}$. PTH, aprotinin and α2-antiplasmin were added at final concentrations of 10^{-9} M, 10^{-5} M, 10^{-5} M and 10^{-5} M respectively. *Significantly different from PTH alone at ($P<0.05$).

Table 6-3 The effects of SP inhibtors on TRAP +ve MNC formation

Treatment	Number of TRAP-positive MNCs per well
$1,25\text{-}(OH)_2D_3$	65 ± 12
$1,25\text{-}(OH)_2D_3$ + CT1399	69 ± 8
$1,25\text{-}(OH)_2D_3$ + Aprotinin	73 ± 13
$1,25\text{-}(OH)_2D_3$ + α2-antiplasmin	62 ± 10

Mouse bone marrow cells were cultured as described in Materials and Methods in the presence of $1,25\text{-}(OH)_2D_3$ (10^{-8} M). Aprotinin, α2-antiplasmin or CT1399 were added at a concentration of 10^{-6} M at the beginning of the culture. After 8 days the cultures were stained for TRAP and the number of TRAP +ve MNCs with over 2 nuclei counted. Results are expressed as mean ± SEM of 6 wells from 2 experiments.

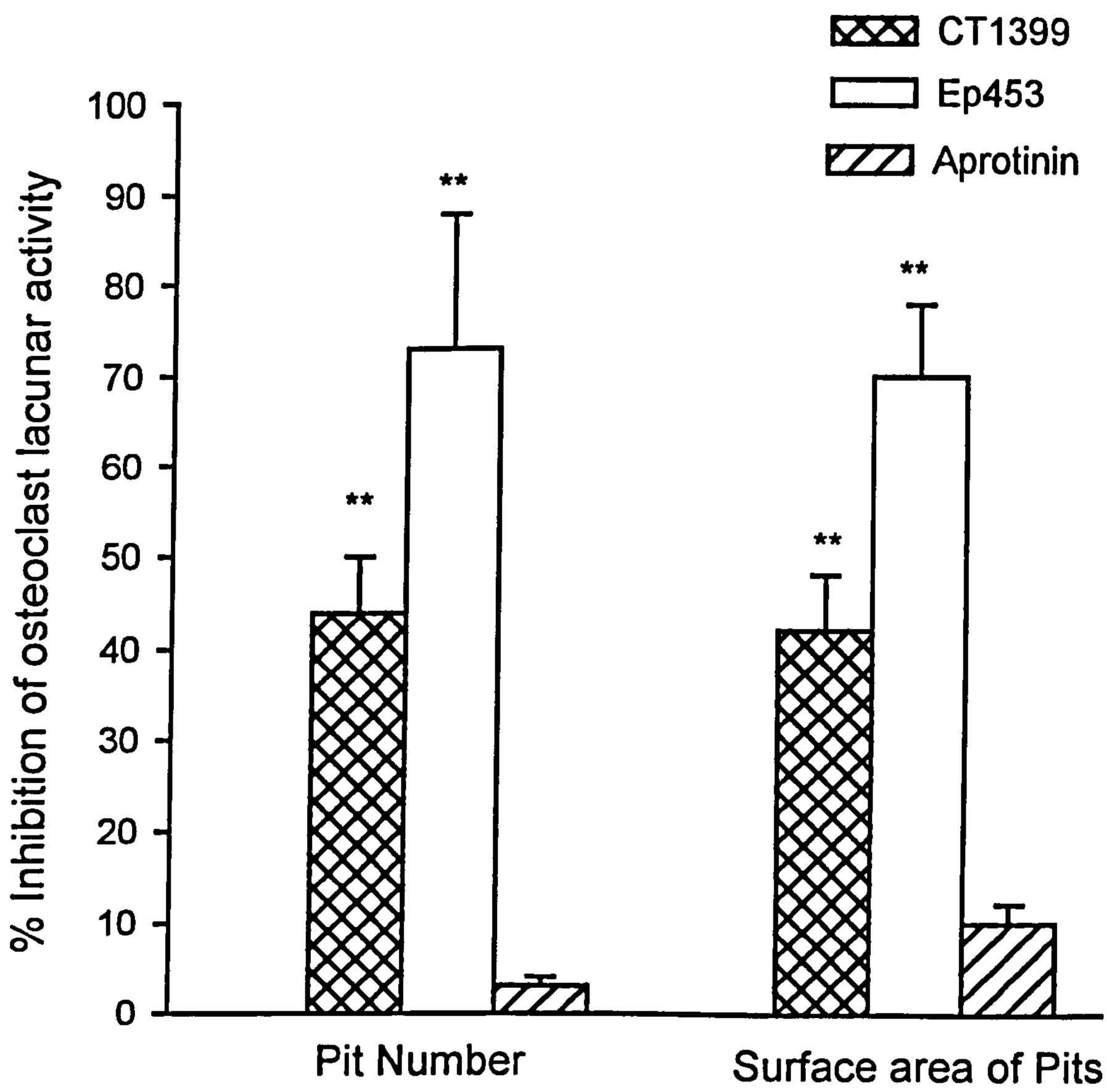


Fig. 6-2 Effects of proteinase inhibitors on the number and the total surface area of mouse osteoclast lacunae.

Each value is the percentage inhibition of osteoclast lacunar resorption arbitrarily set to 100%. The values represent the means ± SEM from five individual experiments representing 15 slices for each variable. The number and surface area of pits on the Ep453 and CT1399 treated slices were significantly different from control (*P< 0.05 and **P< 0.01). The number of lacunae in the control cultures was 1021 (Ep453), 1293 (CT1399) and 879 (aprotinin).

6.3.4 Osteoclast migration and fusion

The role of the PA/plasmin system on osteoclast migration and fusion was examined in noninvaded 17-day-old fetal metatarsal rudiments after 3 and 6 days of culture by means of histomorphometry after TRAP staining.

The effects of the proteinase inhibitors on the number of TRAP+ve cells in both the periosteum and mineralized matrix was determined at the beginning of the experiment and after 3 and 6 days of culture (Fig. 6-3). As the TRAP+ve cells were often multinucleate, especially in the excavating marrow cavity, both TRAP cell profiles and nuclei were counted. The SP inhibitor aprotinin produced a significant reduction of approximately 55% in the invasion of the mineralized matrix by TRAP+ve cells after 3 days with a concomitant accumulation of TRAP+ve cells in the periosteum, thus showing that the SP inhibitors do not stop the formation of new TRAP+ve cells. A similar situation was found for the number of nuclei per TRAP+ve cell. However after 6 days culture with aprotinin the numbers of TRAP+ve cells and nuclei per TRAP+ve cell were similar to the control cultures (Fig. 6-3). In contrast the MMP inhibitor CT1399 completely prevented the invasion of the mineralized matrix by TRAP+ve cells after 3 days with a concomitant increase in the number of TRAP+ve cells in the periosteum. After 6 days CT1399 still produced a significant reduction in the migration of TRAP+ve cells although its effects were incomplete (Fig. 6-3). None of the inhibitors appeared to effect the intrinsic ability of cells to fuse.

6.3.5 Degradation of bone-like matrix

To determine whether the PA system is involved in the degradation of non-mineralized bone matrix, primary osteoblasts were cultured on ^{3}H-labelled bone-like matrix produced by MC3T3-E1 cells. Mouse osteoblasts cultured for 48 h in the absence of serum produced neither basal nor 1,25-$(OH)_2D_3$-stimulated matrix breakdown, but the stimulated osteoblasts produced a 3-fold increase in matrix degradation when plasminogen was added to the cultures. Plasminogen-dependent matrix breakdown was significantly inhibited by the SP inhibitors, aprotinin (10^{-5} M) and α2-antiplasmin (10^{-5} M). Furthermore plasminogen-dependent breakdown was inhibited by the MMP inhibitor, CT1399 (10^{-6} M).

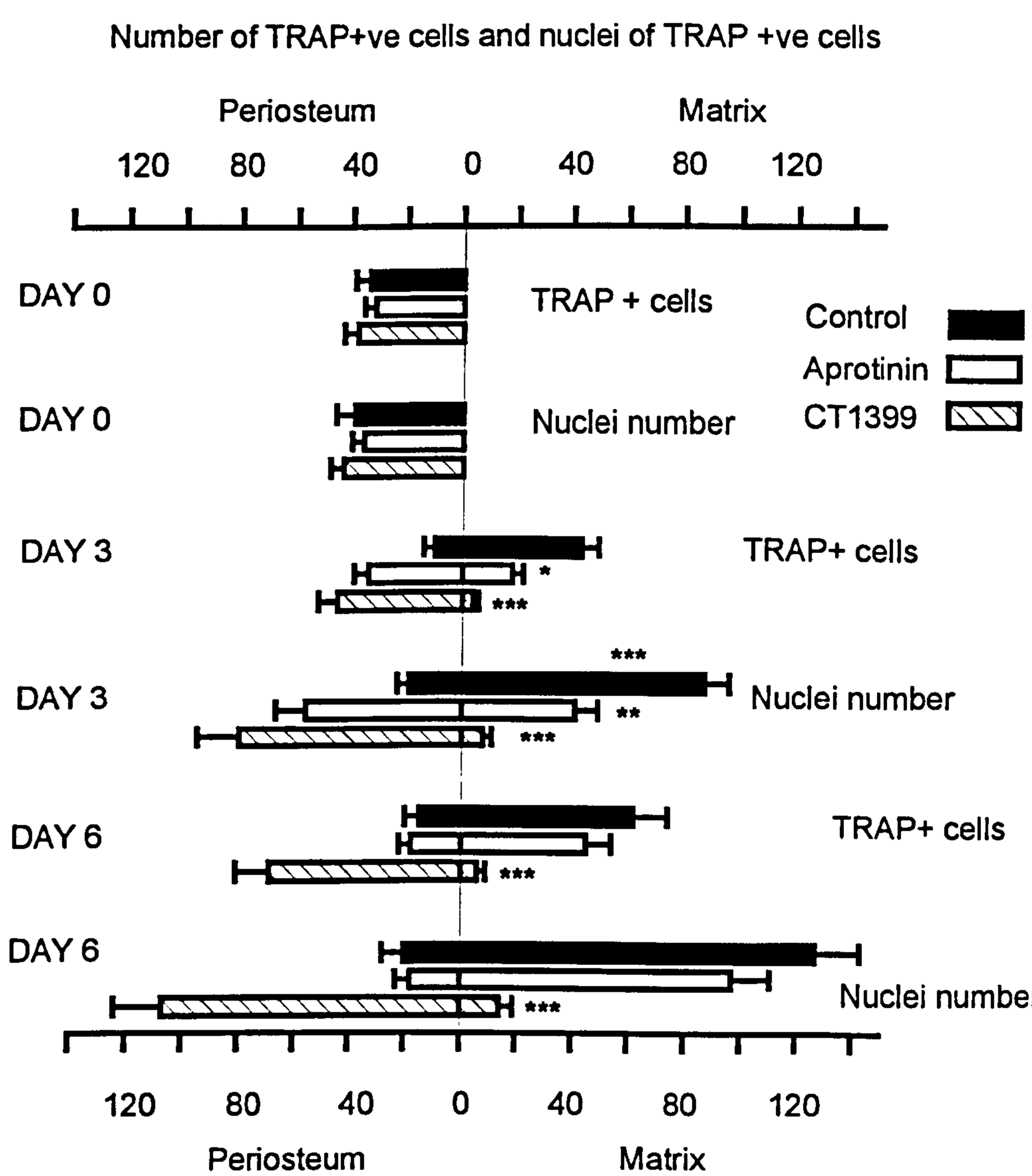


Fig. 6-3. Effects of proteinase inhibitors on the migration of TRAP+ve cells in metatarsal explants.

Metatarsals were obtained from 3 litters of 17-day-old fetal mice. The metatarsal triads of the left limb were cultured in control conditions with 1,25-$(OH)_2D_3$ and those of the corresponding right limbs were cultured in the presence of 1,25-$(OH)_2D_3$ and either CT1399 (10^{-6} M) or aprotinin (10^{-6} M) for the indicated times. The number of TRAP+ve cells and their nuclei localized inside and outside the calcified cartilage (cc) were counted. Counts inside the cc are shown to the right of the '0' axis and those within the periosteum are shown to the left. Each bar (left and right) expresses thus the total numbers in one metatarsal. Counts at day 0, 3 and 6 are the means ± SEM of, respectively, 18, 15, and 21 metatarsals.

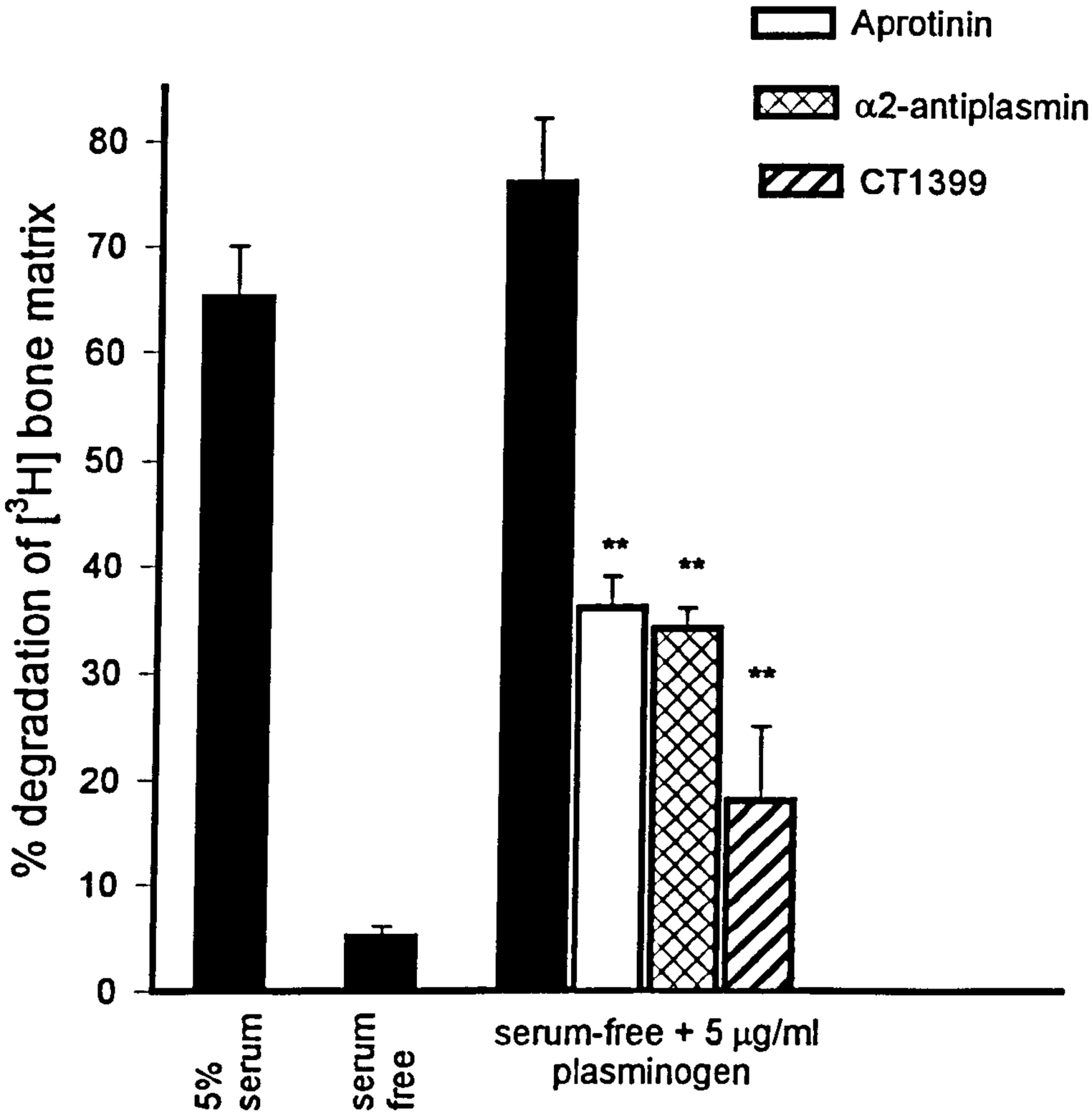


Fig. 6-4. Effects of proteinase inhibitors on the degradation of non-mineralized bone matrix.

Primary mouse osteoblasts were cultured for 48 h on ^{3}H-labelled extracellular matrices and stimulated with 1,25-$(OH)_2D_3$ (10^{-8} M) in the presence of 5% serum, in serum-free medium or in serum-free medium supplemented with 5 µg/ml plasminogen. Aprotinin (10^{-5} M), α2-antiplasmin (10^{-5} M) or CT1399 (10^{-6} M) were added to cultures containing 5 µg/ml of plasminogen. The results are expressed as percentage degradation of ^{3}H-labelled bone matrix. Each bar represents the mean ± SEM of 6 wells. Aprotinin, α2-antiplasmin, and CT1399 inhibited degradation of ^{3}H-labelled bone matrix that was statistically significant compared to the control (**P<0.01).

6.3.6 Degradation of type I collagen films

To confirm a role for the PA system in the degradation of type I collagen, primary osteoblasts were cultured on ^{14}C-labelled type I collagen films for 48 h. Aprotinin dose-dependently inhibited 1,25-$(OH)_2D_3$-stimulated collagen breakdown by mouse osteoblasts (Fig. 6-5). CT1399 produced a complete inhibition whilst the SP inhibitors only produced about a 55% reduction in type I collagenolysis.

6.3.7 Expression of PAs and their inhibitors in osteoblasts

RT-PCR analysis was used to establish expression of mRNA for the PAs and their inhibitors in unstimulated and PTH-stimulated cultures of primary mouse osteoblasts using specific oligonucleotide primers. RT-PCR analysis showed that osteoblasts express mRNA transcripts for tPA, uPA and the type-I receptor for uPA, uPAR-I (Fig. 6-6). RT-PCR analysis using primers for the serine proteinase inhibitors showed expression of PAI-1 and the broad spectrum inhibitor PN-I. The PCR products were of the correct size and the authenticity of the sequences was verified by automated sequencing. RT-PCR of mRNA isolated from PTH-stimulated osteoblasts revealed no difference in expression pattern of the PAs and their inhibitors. The primers for PAI-2 generated PCR fragments between 194 and 420 bp instead of a single band corresponding to the expected size of 205 bp (Fig. 6-6).

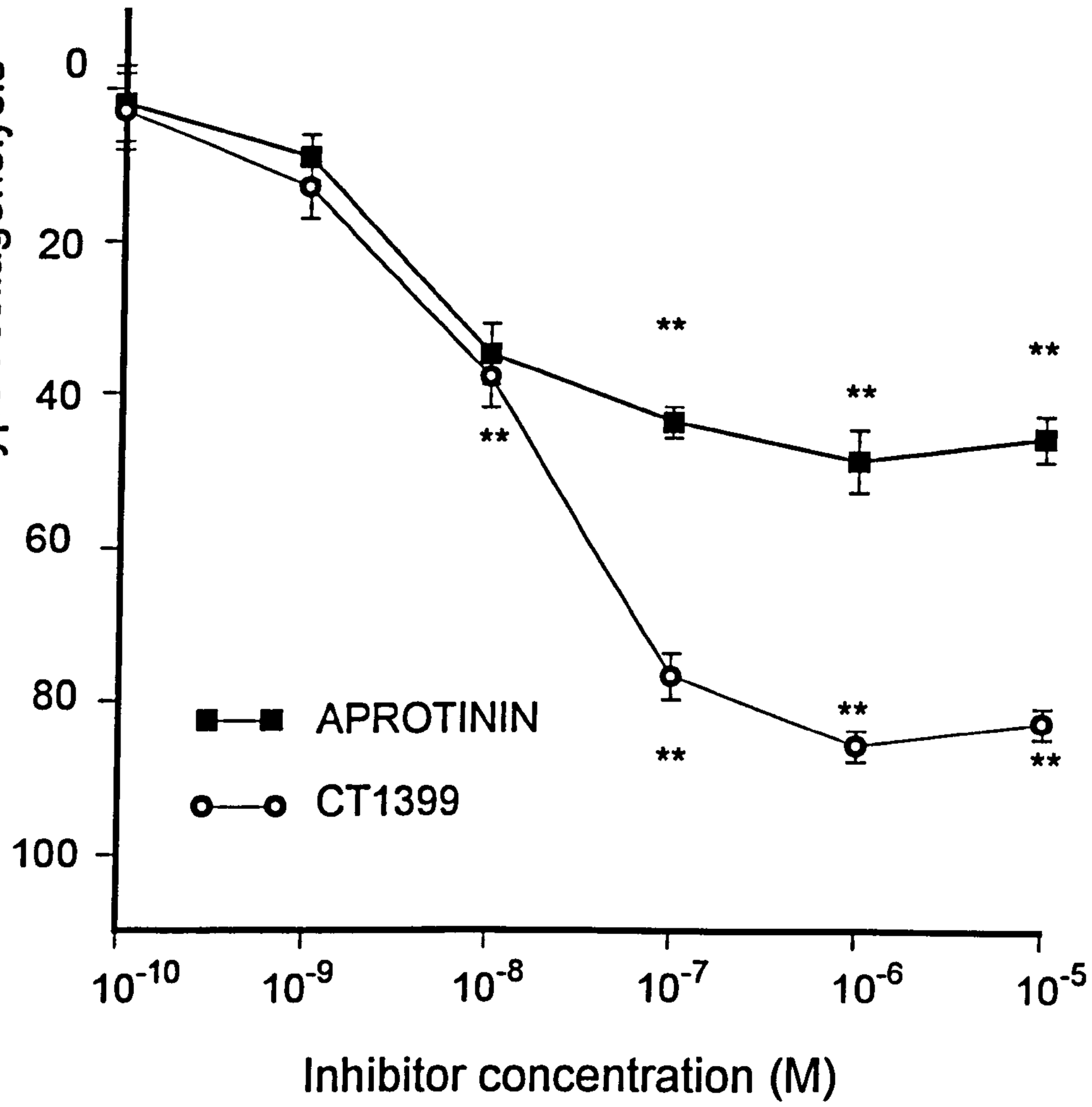


Fig. 6-5. Effects of proteinase inhibitors on the degradation of ^{14}C-labelled type I collagen films by mouse osteoblasts.

Primary mouse osteoblasts were stimulated by 1,25-$(OH)_2D_3$ (10^{-8} M) after 72 hours.The results are expressed as percentage inhibition of 1,25-$(OH)_2D_3$-stimulated ^{14}C release, which was arbitrarily set to 100%. Each point is the mean ± S.E.M. of six wells. The inhibitory effects of CT1399 (10^{-8}-10^{-5} M) and aprotinin (10^{-8}-10^{-5} M) were statistically significant *P<0.05, **P<0.01 compared with control. The percentage release of isotope by 1,25$(OH)_2D_3$-stimulated mouse osteoblasts was 64.7 ± 5.9 which was obtained after subtracting the unstimulated release of isotope (23.7 ± 3.7).

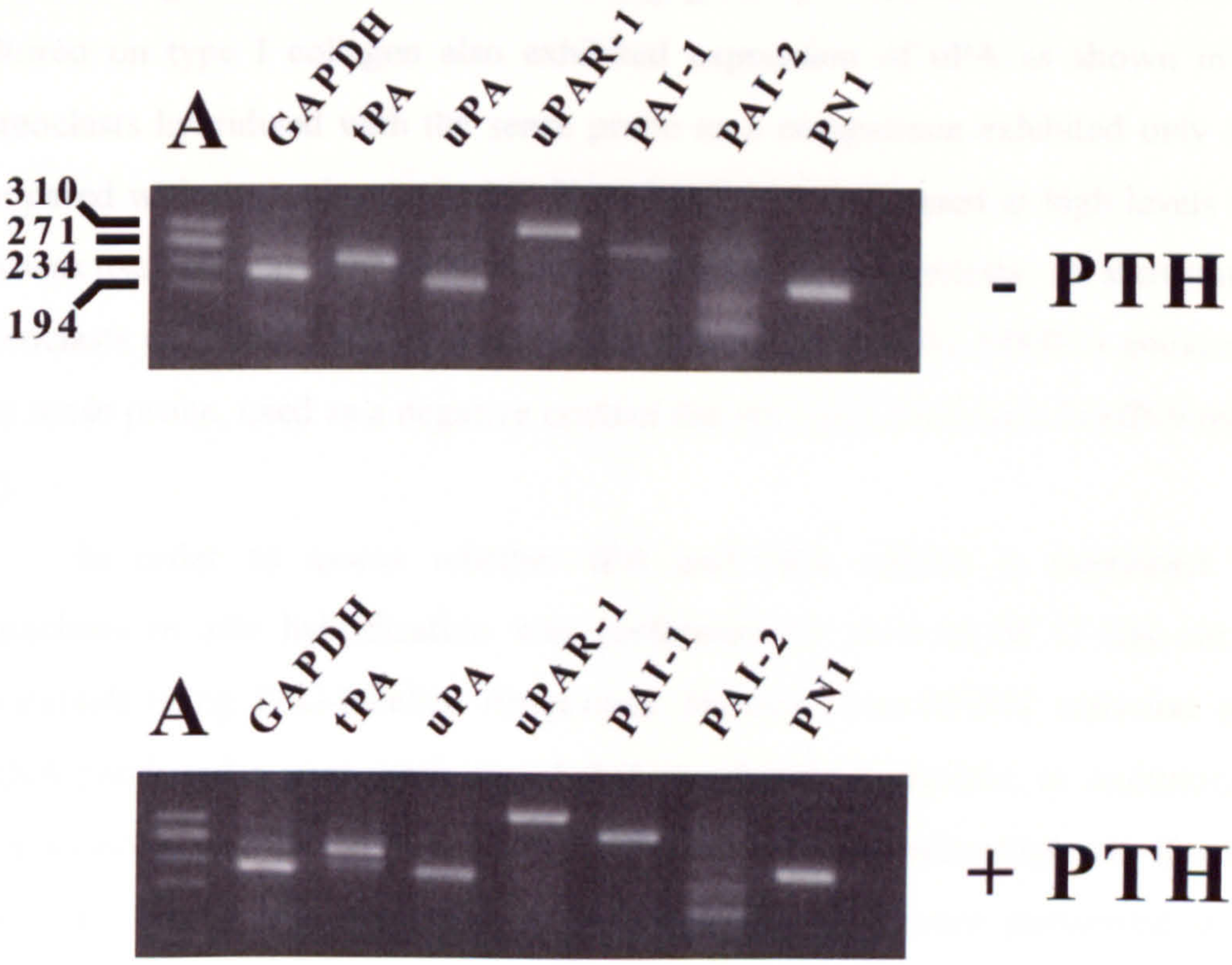


Fig. 6-6. RT-PCR of PAs and their inhibitors osteoblasts.
Primary mouse osteoblasts were cultured as described in materials and methods in 75 cm^2 tissue culture flasks in the presence or absence of 10^{-8} M PTH. Total RNA was isolated and RT-PCR performed with primers specific for: tPA, uPA, uPAR1, PAI-1, PAI-2 and PN1. The housekeeping gene GAPDH was used as a positive control. PCR was performed for 35 cycles in a 100 µl reaction and 10 µl analyzed by gel electrophoresis on a 2% agarose gel. **A**, DNA size marker. No difference was observed in the pattern of mRNA expression between unstimulated and PTH-stimulated mouse osteoblasts.

6.3.8 Expression of PAs in osteoclasts

Expression of mRNA for tPA and uPA were investigated initially by *in situ* hybridization studies on osteoclasts isolated from the long bones of 6-day-old mice. Bone cell suspensions were plated directly onto type I collagen coated glass coverslips in 300 μl of α-MEM and cultured for 3 h to adhere. Figure 6-7A shows a typical multinuceate osteoclast showing expression of tPA mRNA. For comparison the sense probe for tPA was used as a negative control and showed negligible hybridization to tPA mRNA. Osteoclasts cultured on type I collagen also exhibited expression of uPA as shown in figure 6-7C. Osteoclasts hybridized with the sense probe as a comparison exhibited only a weak signal compared with the antisense probe. Since MMP-9 is expressed at high levels in osteoclasts this was used as a positive control and as a marker for osteoclasts. As shown in figure 6-7D osteoclasts exhibited a strong signal when hybridized with the MMP-9 antisense riboprobe. The sense probe, used as a negative control did not hybridized with MMP-9 mRNA (Fig. 6-7E).

In order to assess whether tPA and uPA mRNA is expressed in migrating osteoclasts *in situ* hybridization was performed on sections of 17-day-old fetal mouse metatarsals using DIG-labelled riboprobes. Hybridization of tPA antisense probe to tPA mRNA produced a very weak signal that was barely detectable, in multinucleated and in some mononuclear cells, present in the calcified cartilage, indicating very low expression of tPA. (Fig. 6-8A). As a negative control hybridizations were performed with the sense riboprobe which did not hybridize to mRNA and produced no signal (Fig. 6-8B). In contrast hybridization with the uPA antisense probe produced a stonger signal in cells within the calcified cartilage indicating the presence mRNA for uPA (Fig. 6-8C). MMP-9 has been shown to be important in osteoclast migration in 17-day-old metatarsals (Blavier and Delaisse, 1995). To confirm the presence of osteoclasts within the calcified cartilage adjacent sections were hyridized with MMP-9 antisense riboprobe. As can be seen in figure 6-8E numerous MMP-9 positive osteoclasts were present at the level of calcified cartilage.

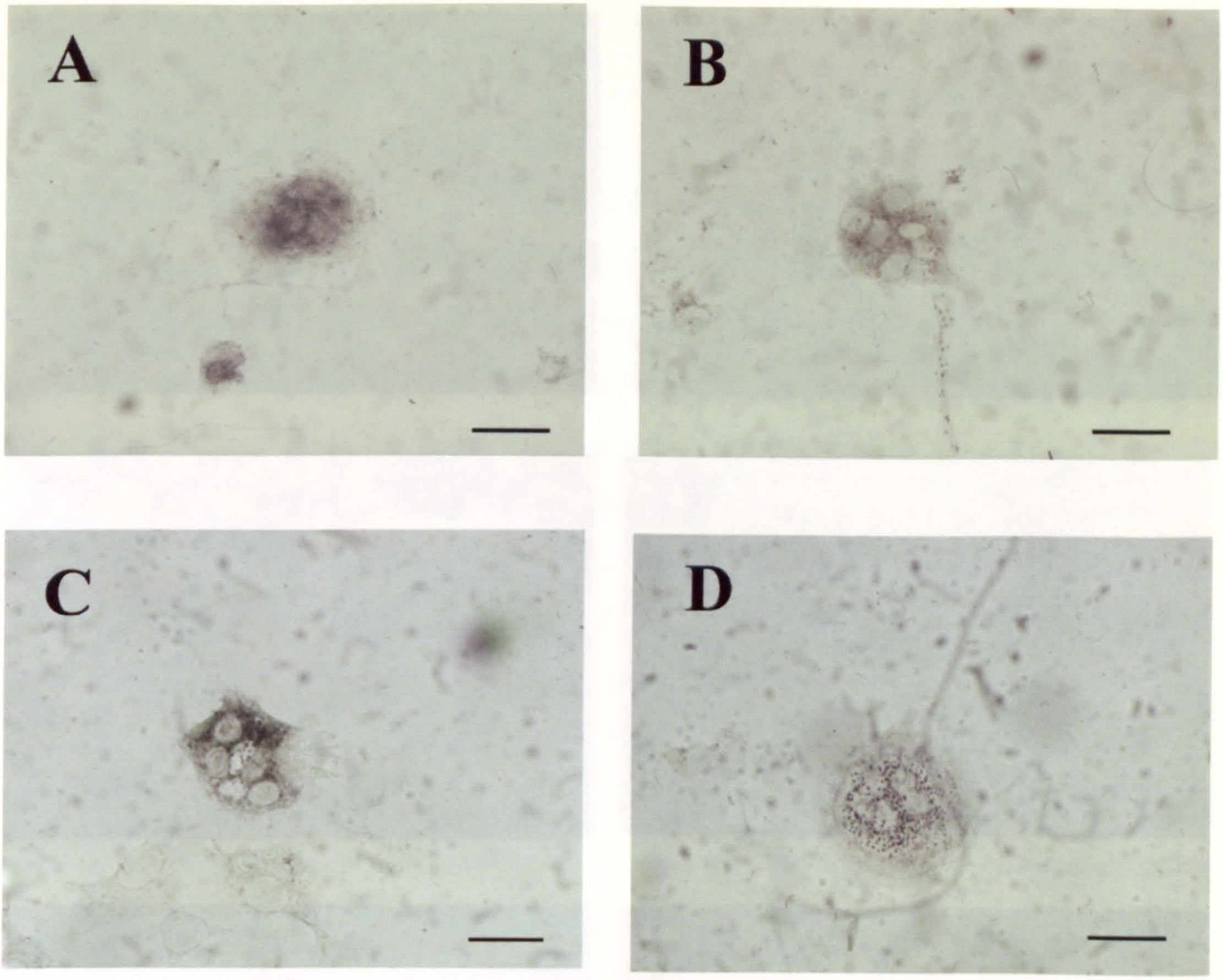


Fig. 6-7. *In situ* hybridization of tPA and uPA in isolated osteoclasts.
Osteoclasts were prepared as described in Materials and Methods and cultured on rat tail type I collagen coated coverslips. Coverslips were hybridized with **A**, tPA antisense riboprobe; **B**, tPA sense riboprobe; **C**, uPA antisense riboprobe; **D**, uPA sense riboprobe; **E**, osteoclasts hybridized with MMP-9 antisense and **F**, MMP-9 sense. Bar = 50 μm. Osteoclasts exhibited expression of tPA and uPA when hybridized with the respective riboprobes and were positive for MMP-9 mRNA expression.

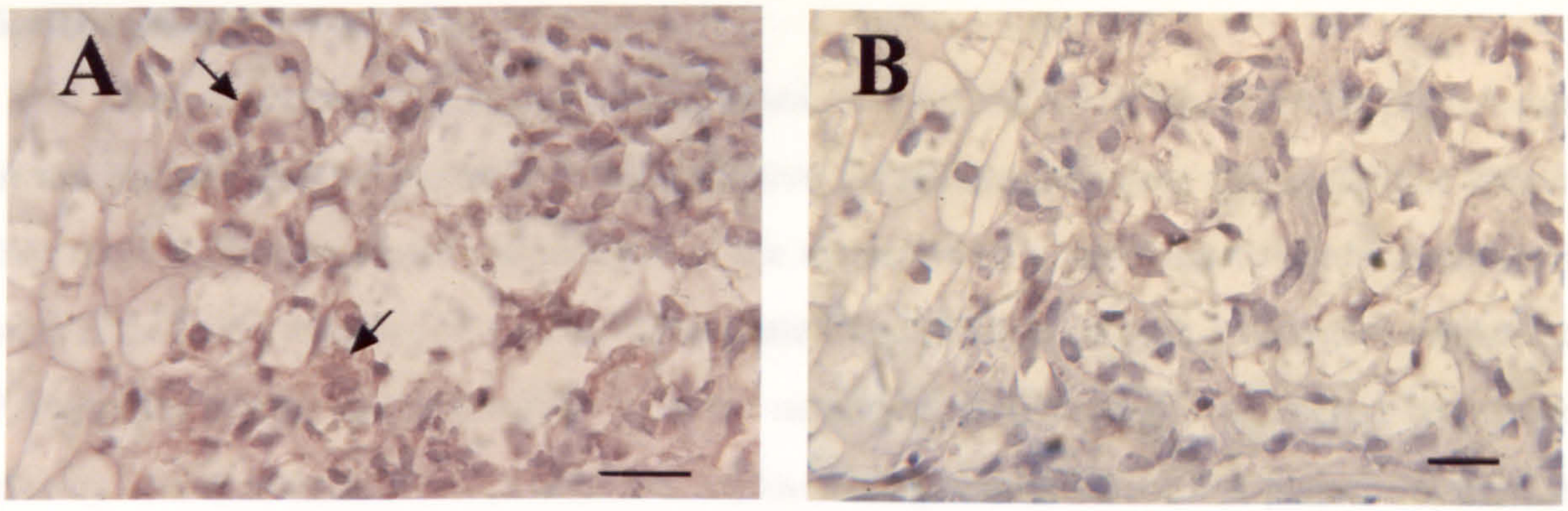


Fig. 6-8. *In situ* hybridization studies of tPA and uPA in 17-day-old fetal mouse metatarsals.

Metatarsal explants were dissected and cultured as described in Materials and Methods. Sections were hybridized with A, tPA antisense riboprobe; B, tPA sense riboprobe; C, uPA antisense riboprobe; D, uPA sense riboprobe; E, adjacent section hybridized with MMP-9 antisense riboprobe; F, MMP-9 sense riboprobe. Metatarsal explants exhibited a weak signal when hybridized with tPA antisense riboprobe and exhibited expression of uPA in cells which also exhibit expression of MMP-9. Bar = 50 μm.

6.4 Discussion

The data presented in this study demonstrate that the PA/plasmin system plays a role in the bone resorption cascade. Using selective proteinase inhibitors this study has shown that the PA/plasmin system is involved in the migration of osteoclasts to future resorption sites and is also involved in osteoblast-mediated degradation of type I collagen, whilst the PA/plasmin system is not involved in either osteoclast formation nor osteoclast resorptive activity. Furthermore the expression of various components of the PA/plasmin system in primary mouse osteoblasts and osteoclasts has been demonstrated.

Aprotinin is a small extremely stable peptide that reacts rapidly with plasmin to form high-affinity complexes (with a kd of 10^{-9}-10^{-10} M). In contrast to α_2-antiplasmin and α_1 antitrypsin, the main inhibitor present in plasma, aprotinin also inhibits plasmin when it is bound to the plasminogen/plasmin surface receptors (Stephens *et al.*, 1989 Bizik *et al.*, 1990) found on many cell types (Mignatti *et al.*, 1986).

The neonatal murine calvarial bone resorption assay reflects mainly post-mitotic events, namely the fusion of preosteoclasts, the activation of fully developed mature osteoclasts and osteoblast degradation of collagen fibrils whilst it is virtually independent of proliferation of osteoclast progenitors. This probably explains why the SP inhibitors, aprotinin and α_2-antiplasmin were less effective at preventing calvarial resorption than the MMP inhibitor, CT1399, since SP activity is limited to osteoblast-mediated degradation of the osteoid layer in this culture system.

It is widely believed that prior to osteoclastic resorption, the bone surface is freed of a thin investing layer of non-mineralized collagen fibrils. Although, osteoblast-derived MMPs, in particular collagenase, have been implicated in this process, it has been suggested that the PA/plasmin system may also be involved in this process (Thomson *et al.*, 1989). The findings that osteoblast-mediated degradation of both type I collagen and bone-like matrix is dependent on the presence of plasminogen and that the SP inhibitor, aprotinin partially prevented osteoblast-mediated degradation of these substrates supports the concept that the PA/plasmin system is involved in this aspect of the bone resorption process. Consistent with a role for the PA/plasmin system in osteoid degradation it has been shown that degaradation of nonmineralized matrix by cocultures of osteoblasts and osteoclasts is decreased by combined inactivation of uPA and tPA genes (Daci *et al.*, 1999). Furthermore, the demonstration in this study that osteoblasts express tPA and uPA is in

accordance with previous studies that have shown that a variety of bone resorbing agents upregulate PA activity in osteoblasts (Hamilton *et al.*, 1984;1985). Although the precise mechanism of action of the PA/plasmin system in collagen degradation has not been demonstrated in this study it is known that plasmin will activate MMPs, particularly prostromelysin-1 and -2. In concert, plasmin and stromelysins then activate other osteoblast derived MMPs (Meikle *et al.*, 1992) in particular collagenase and gelatinase B (Murphy and Knauper, 1997) which may be directly responsible for the type I collagen degradation (Hill *et al.*, 1995).

The finding from this study that aprotinin prevented the migration of osteoclasts from the periosteum to the mineralized matrix in 17-day-old fetal metatarsals is similar to findings of Hoekman *et al.* (1992) who demonstrated that tPA stimulated osteoclastic resorption in these explants an event that is indicative of osteoclast migration to the calcified matrix. Furthermore, the results presented here demonstrate expression of uPA in fetal metatarsal explants by *in situ* hybridisation. Leloup *et al.* (1994) also demonstrated that uPA is the main PA present in extracts of cultured fetal mouse metatarsals. Although, Leloup *et al.* (1994) reported that inhibitors of plasmin did not influence metatarsal bone resorption they found that when the explants were cultured in serum depleted of plasmin inhibitors there was enhanced bone resorption suggesting that the PA/plasmin system may be involved. Furthermore LeLoup *et al.*, (1996) subsequently demonstrated that in mice with an inactivated uPA gene, bone resorption was reduced at the commencement of culture in the metatarsal explants whereas inactivation of the tPA gene had no effect on bone resorption in fetal metatarsal explants. A combined inactivation of both uPA and tPA in these studies did not further inhibit bone resorption at the commencement of culture.

The inhibitory activity of aprotinin against the invasion of preosteoclasts and the degradation of type I collagen by osteoblasts is comparable with the inhibitory activity of aprotinin in several other culture models in which it contributed to establish the role of the PA/plasmin system in either invasion or the degradation of extracellular matrices (Mignatti *et al.*, 1986; Cajot *et al.*, 1989; Quax *et al.*, 1991).

The expression of tPA and uPA has been identified in osteoclasts both at the message level by RT-PCR (Yang *et al.*, 1997), and at the protein level by immunocytochemistry (Grills *et al.*, 1990). Furthermore Yang *et al.* (1997) demonstrated expression of uPAR1, by *in situ* hybridisation, in osteoclasts cultured at pH 6.9 although at

pH 7.3 little expression was observed. However, results from this study demonstrate that aprotinin had no inhibitory effect on osteoclast lacunar resorptive activity suggests that, at least under these in vitro conditions used in this study, the PA/plasmin system is not directly involved in this aspect of the resorption cascade. These findings are consistent with the results reported by Daci *et al.* (1999) in which osteoclasts derived from mice with a combined inactivation of both uPA and tPA were still able to resorb dentine. Also Hoekman *et al.* (1992) found that tPA had no effect on bone resoprtion in 17-day-old fetal radii that contain mature osteoclasts. Nonetheless, there are several potential roles for the osteoclast derived PAs in bone. These include the activation of latent proteases (Kwaan, 1992), activation of latent growth factors (Martin *et al.*, 1993; Lalou *et al.*, 1994), and a nonproteolytic role as a mitogenic agent (Kirchheimer *et al.*, 1987; Rabbani *et al.*, 1990). The activation of various proenzymes by the PA system in osteoclasts, including enzymes that degrade extracellular matrix proteins such as prostromelysin and procollagenase, could aid in the resoprtion of bone (Delaisse and Vaes, 1992). Thus, since there are various proteolytic enzymes that appear to have overlapping functions in the activation of proenzymes or paracrine factors involved in bone resorption, the PA system may be one of several redundant mechanisms involved in this process. Another function of the PA system in osteoclasts may be to activate paracrine factors involved in the regulation of bone remodelling (Martin *et al.*, 1993). Plasmin has been shown to dissociate IGF-I from its binding protein in human osteosarcoma cells and to activate the IL-1β precursor and latent TGF-β (Martin *et al.*, 1993; Kwaan, 1992).

In this study aprotinin and α2-antiplasmin had no effect on formation of TRAP +ve MNCs. Similarly, Daci *et al.* (1999) reported that cocultures of primary osteoblasts and bone marrow cells from wild type mice were able to generate TRAP +ve MNCs in the presence of aprotinin. Also cocultures of primary mouse osteoblasts and bone marrow cells derived from mice with an inactivation of uPA, tPA or a combined inactivation of both were capable of forming TRAP +ve MNCs when stimulated with 1,25-$(OH)_2D_3$ (Daci *et al.* 1999).

7. Conclusions and Considerations for Future Work

7.1 Osteoblast Survival

The results from this investigation provide new insights into the cellular mechanisms by which certain growth factors present in bone matrix, notably the IGFs, FGF and PDGF may co-operatively effect osteoblast survival. The findings from chapter 2 demonstrate that bone matrix derived growth factors, IGF-I and IGF-II, increased osteoblast survival *in vitro* and this was synergistically enhanced by PDGF and bFGF. The importance of FGF in osteoblast survival and bone development is highlighted by craniosynostosis disorders in which there is premature closure of the cranial sutures. This has been proposed to be a result of an inhibition of osteoblast apoptosis (Rice *et al.*, 1999) due to defective FGF signalling. Human craniosynostosis disorders have been linked to mutations in the FGF receptor-1 and -2 genes (Muenke and Schell, 1995). Interestingly osteoblasts derived from patients with syndromic craniosynostosis have been shown to inhibit the apoptotic rate of normal human osteoblasts through the production of soluble factors (Dry *et al.*, 2001). In contrast to the results presented in this thesis it has been shown that FGF can also induce apoptosis in osteoblast cultures undergoing differentiation in the presence of ascorbic acid and β-glycerophosphate (Mansukhani *et al.*, 2000).

The importance of IGF-I *in vivo* has been demonstrated by targeted overexpression of IGF-I in osteoblasts of transgenic mice (Zhao *et al.*, 2000). In these mice there was increased trabecular bone volume without an increase in osteoblast number. Although this increase in trabecular bone volume was ascribed to increased osteoblast activity, IGF-I may also have increased osteoblast survival. It has been shown that glucocorticoids also decrease levels of IGF-I (Delaney and Canalis, 1995). Thus in glucocorticoid induced osteoporosis decreased bioavailability of IGF-I may effect osteoblast activity and survival. The main focus of future work will involve elucidating the signal transduction pathways by which these locally produced growth factors increase osteoblast survival. This may then provide a target for therapeutic drugs to mimic the actions of these growth factors thereby increasing osteoblast lifespan and subsequent bone mass.

Recently it has been shown that the plasma proteinase inhibitors α1-proteinase inhibitor, α1-antichymotrypsin and α2-macroglobulin act as antiapoptotic factors for human

vascular smooth muscle cells (Ikari *et al.*, 2001). Given that the combinations of growth factors investigated in the course of this thesis was insufficient to achieve a level osteoblast survival comparable to survival in the presence of serum it would have been interesting to investigate the role of proteinase inhibitors on osteoblast survival. Other proteinase inhibitors have recently been shown to be important determinants of cell survival; of particular interest is the demonstration that TIMP-3 promoted apoptosis in smooth muscle cells (Bond *et al.*, 2000). This activity was associated with the metalloproteinase inhibiting activity of TIMP-3. TIMP-3 exhibits a unique ability to inhibit members of the ADAM family (Amour *et al.*, 1998; Amour *et al.*, 2000; Kashiwagi *et al.*, 2001) and inhibition of proteolytic shedding by TIMP-3 may effect cell survival. The serine proteinase inhibitor, PAI-2, may be important in protecting cells from TNF-induced apoptosis at inflammatory sites (Dickinson *et al.*, 1995) since it has been shown that HeLa cells overexpressing PAI-2 are protected from cell death induced by TNF.

Cell-cell contact may also be important to osteoblast survival. Expression of N-cadherin has been demonstrated in immortalized human neonatal calvarial cells and expression of N-cadherin was upregulated by FGF-2 (Debiais *et al.*, 2001). Furthermore N-cadherin-mediated cell-cell interactions in melanoma cells inactivate the proapoptotic factor BAD (Li *et al.*, 2001). Whether a similar mechanism operates in osteoblasts remains to be investigated.

Although the results from this investigation show that antioxidants promote osteoblast survival *in vitro* the importance of reactive oxygen species to osteoblast survival *in vivo* is uncertain. It has been shown *in vitro* that TRAP can generate reactive oxygen species and that cells overexpressing TRAP produce higher amounts of intracellular reactive oxygen species (Halleen *et al.*, 1999). Antioxidants thus may be important in protecting osteoblasts from reactive oxygen species produced by osteoclasts during bone remodelling. Interestingly, it has been demonstrated that human fetal osteoblasts produce glutathione peroxidase and selenoproteins which possess antioxidant properties (Dreher *et al.*, 1998).

Production of TNFα by T-cells has been shown to play a key role in bone loss associated with rheumatoid arthritis and TNFα is also important in the pathogensis of bone loss associated with tumour osteolysis and periodontal disease. The findings presented in chapter 2 that TNF-α promotes osteoblast apoptosis may further contribute to the current understanding of the mechanisms involved in these diseases. Indeed, it has been shown that

culture supernatants from activated T-cells induced apoptosis in primary human osteoblasts and that the bisphosphonate, etidronate, inhibited the production of pro-apoptotic factors by T-cells (Abe *et al.*, 2000).

7.2 Importance of IL-11 in bone cell function

Studies in chapter 4 highlight the importance of IL-11 as a cytokine that influences osteoblast activity and osteoclast formation and function. Although IL-11 may play a role in normal bone metabolism, the importance of IL-11 as a cytokine that stimulates osteoclast formation may be more relevant to pathological conditions characterized by bone loss. Previous studies have implicated IL-11 as an important cytokine in tumour promoted osteolysis. The human melanoma cell line, A375M, and the breast cancer cell line, MDA-MB-231, have been shown to form osteolytic bone metastases *in vivo* and both cell lines produce IL-11 by themselves and stimulate IL-11 production from osteoblasts (Morinaga *et al.*, 1997). The results from chapter 4 may be relevant to understanding cellular mechanisms involved in recruitment of osteoclasts in osteolytic bone diseases. Furthermore, the stimulation of osteoblast mediated osteoid degradation by IL-11 may exacerbate progression of tumour osteolysis by predisposing mineralized bone surfaces to bone resorption by osteoclasts. IL-11 may also be involved in mechanisms of bone remodelling in osteoarthritis and rheumatoid arthritis since osteoblasts and bone marrow stromal cells from patients with these diseases constitutively secrete IL-11 (Lisignoli *et al.*, 2000). Kim *et al.* (1997) demonstrated that IL-1 stimulates the production of IL-11 by human bone marrow stromal cells, raising the possibility that IL-11 may play a key role in bone loss associated with estrogen deficiency. However, stimulation of IL-11 by IL-1 was not inhibited 17 β-estradiol suggesting that IL-11 may play only a minor role in bone resorption in estrogen deficiency.

The observation that IL-11 stimulates osteoblast-mediated type I collagen degradation indicate that IL-11 may play a major role in regulating MMP expression in osteoblasts. Further studies will be required to identify which MMPs are expressed by mouse osteoblasts in response to IL-11 and whether this cytokine is responsible for enhancing osteoblast MMP synthesis in response to stimulation by PTH, IL-1, and 1,25-$(OH)_2D_3$. Interestingly, IL-6 with sIL-6R has been shown to stimulate mRNA expression of MMP-2, -3, -9 and -13 in cultured mouse calvaria (Kusano *et al.*, 1998). In the same study

IL-1 also increased expression of these MMPs but to a greater extent. Future work will also be neccessary to establish whether IL-11 regulates the expression of members of the ADAM family in mouse calvarial osteoblasts.

Although osteoclasts have been shown to express IL-11 receptor transcripts (Romas *et al.*, 1996) the results presented in chapter 4 show that IL-11 did not stimulate bone resorption by isolated osteoclasts. Other investigators have shown that IL-11 does not influence osteoclast survival and antibody to gp130 does not effect lacunar resorption by osteoclasts. Interestingly the data presented in chapter 4 suggests that IL-11 may alter the balance between MMPs and TIMPs. Future investigations will investigate the regulation of MMP/TIMP expression in bone cells by IL-11 to determine whether an alteration in this balance is responsible for matrix degradation. It will also be interesting to determine whether IL-11 effects the expression of members of the plasminogen activator/inhibitor pathway, which may also be important in osteoclast migration in the 17-day-old fetal metatarsal model employed in this study.

The precise involvement of IL-11 in normal bone remodelling will be revealed by generating knockout mice. Mice with a targeted mutation in the IL-11 receptor display normal hematopoiesis although the effects on bone turnover have not been studied (Nandurkan *et al.*, 1997).

7.3 Role of ADAMs and serine proteinases in bone cells

The data presented in chapter 5 demonstrates a role for ADAM-12 in the formation of osteoclasts. Furthermore, the requirement of ADAM proteins in osteoclast formation provides a useful target for intervention by therapeutic agents in the treatment of pathological disease states leading to bone loss. The addition of ODNs to bone marrow cultures had an inhibitory effect on osteoclast formation. Furthermore, recombinant ADAM-12 cysteine rich domain/GST fusion protein inhibited osteoclast formation from MDBM cells stimulated with RANKL and M-CSF. To eliminate any possibility that the inhibitory effect was due to the cysteine rich domain being fused to GST further investigations will need to be performed using purified A12/cys that has been cleaved from the GST using thrombin agarose. It would have been interesting to have performed pulse experiments by adding A12/cys at different stages of culture to see what effect this had on osteoclast formation. Construction of a mutant cysteine rich domain by deleting the

hydrophobic sequence or introducing point mutations by oligonucleotide-site directed mutagenesis may prove useful in elucidating which region of the cysteine rich domain is responsible for its biological activity.

It has been demonstrated that recombinant disintegrin/cysteine-rich domain of mouse ADAM-12 is an active cell adhesion domain by supporting adhesion to C2C12 myoblasts and to NIH 3T3 fibroblasts (Zolkiewska, 1999). Other studies have shown that the recombinant disintegrin domain on its own can bind to $\alpha_9\beta_1$ integrin (Eto *et al.*, 2000) and recombinant cysteine-rich domain binds to syndecans (Iba *et al.*, 2000) and IGFBP-3 (Shi *et al.*, 2000) in the case of human ADAM 12-S. The main focus for future work will involve construction of expression libraries representative of MDBM cells or osteoblasts and screening them with biotinylated disintegrin, cysteine-rich or disintegrin/cysteine-rich domain to identify potential targets for these domains. It would also be interesting to add recombinant disintegrin domain to bioassays for osteoclast formation to see if it also has an inhibitory effect.

A number of questions remain to be answered with regard to the role of ADAMs proteins in osteoclast formation. Although the results from this study demonstrate a role for ADAM-12 in osteoclast formation it is not clear whether ADAM-12 is involved in cell fusion *per se* or whether it is involved in a pre-fusion step. Such a pre-fusion activity may involve mediating a cell-cell interaction between osteoclast precursors or between osteoblasts and osteoclast precursors. Interestingly osteoblasts express syndecans -2 and -4 (Modrowski *et al.*, 2000); thus one possible scenario is an interaction between osteoblasts and ADAM-12 expressed on osteoclast precursors. Although expression of ADAM-9 was not demonstrated by *in situ* hybridization other investigators have shown expression of ADAM-9 in osteoclasts by RNAse protection assay (Inoue *et al.*, 1998). Also, the cysteine rich domain of ADAM-9 contains a hydrophobic fusion peptide sequence. It may be that ADAM-12 interacts with other ADAM family members such as ADAM-9 or ADAM-19 in a similar way to the heterodimerization that occurs between ADAM-1 and ADAM-2 to form fertilin. In such a case one ADAM would mediate cell-cell adhesion between osteoclast precursors whilst the other ADAM would mediate fusion of the plasma membranes. To investigate this it will be necessary to do immunoprecipitation and western blotting with antibodies specific for different ADAM family members.

Disruption of ADAM-12 expression in cultures of primary mouse osteoblasts had no effect on osteoblast differentiation as assayed by mineralized nodule formation. It is intrigueing that previous studies have shown that human ADAM 12-S can regulate IGFBP-3 and -5 levels in pregnancy serum (Shi *et al.*, 2000; Loechel *et al.*, 2000). Also IGFBP-5 is degraded in conditioned medium from differentiating MC3T3-E1 cells by a 97 kDa protease that is not an MMP (Thraikill *et al.*, 1995). Although mouse ADAM 12-S has not been cloned it is conceivable that membrane bound ADAM-12 could be shed from the plasma membrane. It would be interesting to see if IGFBP-5 is degraded in conditioned medium of osteoblasts transfected with ADAM-12 antisense constructs. Regulation of IGFBP levels by ADAMs would provide a cellular mechanism for influencing the bioavailability of IGFs to osteoblasts and thus affecting osteoblast proliferation and survival.

Addition of antisense oligodeoxnucleotides specific for ADAM-12 had little effect on osteoclast activity as assessed by lacunar resorption. Although no assessment of osteoclast survival was made in these cultures, it seems unlikely that disruption of ADAM-12 by antisense oligodeoxynucleotides effects osteoclast survival. It would be interesting to assess the effects of disruption of other ADAMs such as ADAM-9 and -19 on osteoclast survival. Since TIMP-3 preferentially inhibits proteolytic shedding by members of the ADAM family it would be interesting to investigate the effect TIMP-3 on survival of mature osteoclasts and on osteoclast generation.

Although the results presented here demonstrate expression of ADAM-12 in 17-day-old metatarsals; specific inhibitors of ADAM-12 will be required to investigate whether ADAM-12 is involved in osteoclast migration in this *in vitro* system. It seems more likely that serine proteinases may be more important in osteoclast migration. The findings from chapter 6 demonstrate that inhibitors of serine proteinases inhibit osteoclast migration over three days in mouse metatarsal explants, thus implicating the PA/plasmin pathway in osteoclast migration. The findings from chapter 6 also demonstrate a role for the PA/plasmin pathway in the degradation of nonmineralized matrix. However *in vivo* studies demonstrate that mice deficient in uPA and tPA have no obvious skeletal defects suggesting that the PA/plasmin system is not a key player in bone remodelling (Leloup *et al.*, 1996; Daci *et al.*, 1999). Future work will focus on regulation of expression of plasminogen activators and their natural inhibitors PAI-I and PAI-II in 17-day-old metatarsals.

It seems that proteinases other than PAs may have a critical role in bone remodelling as suggested by genetic disorders giving rise to an abnormal bone phenotype. Mutations in the cathepsin K gene have been linked to pycnodysostosis, characterized by osteosclerosis (Ho *et al.*, 1999). More recently, mutations of MMP-2 in consanguineous families have been shown to cause osteolyses characterized by crippling arthritic changes and marked osteoporosis (Martignetti *et al.*, 2001). The mechanism by which MMP-2 deficiency results in an osteolytic disorder in these cases is unclear but may involve defective bone formation resulting from incomplete extracellular matrix degradation.

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9. Appendices

9.1 Appendix 1

9.1.1 Tyrodes Solution

KCl	200 mg/L
NaCl	800 mg/L
NaHCO3	1 g/L
NaH2PO4.H2O	50 mg/L

The above chemicals were dissolved in 800 ml of deionized water and the pH adjusted to 7.4 with 1 M NaOH. The solution was then made up to 1L with deionized water.

9.1.2 Phosphate Buffered Saline

NaCl	8.0 g/L
KCl	0.2 g/L
Na2HPO4	1.44 g/L
KH2PO4	0.24 g/L

The above chemicals were dissolved in 800 ml of deionized water and the pH adjusted to 7.4. The solution was then made up to 1L with deionized water.

9.1.3 Preparation of 50x Tris-Acetate-EDTA

Tris base	242 g/L
Glacial acetic acid	57.1ml/L
0.5 M EDTA	100 ml/L

Tris base was dissolved in 800 ml of deionized water and the glacial acetic acid and EDTA added. The buffer was made up to 1.0L with deionized water.

9.1.4 Tween Lysis Buffer

N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)	13.02 g/L
Ethylenediaminetetraacetic acid (EDTA)	0.37 g/L
Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA)	0.95 g/L
NaCl	8.76 g/L
Dithiothreitol (DTT)	0.15 g/L
Tween-20	1.0 ml/L
NaF	0.04 g/L
Na3VO4	0.02 g/L

HEPES was dissolved in 800 ml of deionised water and the pH adjusted to pH 8.0 with 1 M NaOH. The other chemicals were then added, the pH checked and the solution made up to 1 L with deionised water.

9.1.5 Reagents for SDS-PAGE electrophoresis

9.1.5.1 Preparation of Resolving gel buffer (1.5 M Tris.Cl pH 8.8)

Tris base	181.5 g/L

Tris base was dissolved in 800 ml of deionized water and the pH adjusted to 8.8 with 10 M HCl. The buffer was then made up to 1 L with deionized water.

9.1.5.2 Preparation of Stacking gel buffer (0.5 M Tris.Cl pH 6.8)

Tris base	60.5 g/L

Tris base was dissolved in 800 ml of deionised water and the pH adjusted to 6.8 with 10 M HCl. The buffer was then made up to 1 L with deionized water.

9.1.5.3 Preparation of 5 x electrophoresis buffer

Tris base	15.14 g/L
Glycine	72.07 g/L
SDS	5.0 g/L

The above chemicals were dissolved in 1L of deionized water

9.1.5.4 Preparation of 3x sample buffer (10 ml)

1 M Tris.HCl pH 6.8	1.85 ml
10% SDS	0.6 g
10% Bromophenyl blue	75 µl
Glycerol	3.0 ml
2-Mercaptoethanol	300 µl

The above chemicals were combined in a fume cupboard and the volume made up to 10 ml with deionized water.

9.1.5.5 Preparation of coomassie blue destain solution

Methanol	200 ml/L
Acetic acid	100 ml/L
Deionized water	700 ml/L

9.1.5.6 Preparation of 12% resolving gel

30% (w/v) Acrylamide: 0.8% (w/v) Bis-acrylamide stock (37.5:1)	8.4 ml
Resolving gel buffer	5.0 ml
Deionized water	6.25 ml
10% SDS	200 µl
N,N,N',N'-Tetramethylethylenediamine (TEMED)	9.0 µl
10% Ammonium persulphate (APS)	200 µl

The acrylamide (National Diagnostics Ltd, Hull, UK), resolving gel buffer, SDS and water were combined in a universal. TEMED and APS were added and the mixture pipetted

between glass plates of the SDS-PAGE apparatus followed by addition of water saturated isobutanol. The gel was allowed to polymerise for 1 h and the isobutanol washed off.

9.1.5.7 Preparation of stacking gel

30% (w/v) Acrylamide: 0.8% (w/v) Bis-acrylamide stock (37.5:1)	0.7 ml
Stacking gel buffer	1.25 ml
Deionized water	2.95 ml
10% SDS	50 µl
TEMED	5.0 µl
10% APS	50 µl

The above chemicals were combined in a universal. After washing away the water saturated isobutanol from the resolving gel the stacking gel was pipetted on top followed by insertion of the comb.

9.1.6 Assay for secreted placental alkaline phosphatase

9.1.6.1 Preparation of diethanolamine (DEA) buffer

1 M diethanolamine	105.4 g/L
0.28 M NaCl	16.36 g/L
0.5 M $MgCl_2.6H_2O$	0.102 g/L

The above chemicals were dissolved in 800 ml of deionized water and the pH adjusted to 9.85 with concentrated HCl. The solution was made up to 1L with deionized water.

9.1.6.2 Preparation of 20x p-nitrophenylphosphate (PNPP, 100 mM)

PNPP (742 mg; Sigma) was dissolved in 20 ml of DEA buffer and stored at -20°C protected from light. For the assay 1 X PNPP (5 mM) was prepared in DEA buffer and 1 ml added to 50 µl of medium sample that had been heat inactivated at 65°C for 45 min. The absorbance was measured at 405 nm when a yellow colour had developed. SPAP activity was expressed in units where 1 unit is defined as the amount of SPAP that will hydrolyse 1.0 nmol of p-nitrophenylphosphate per minute.

9.1.7 Preparation of neutral buffered formalin

Formaldehyde	100 ml
Na_2HPO_4	16 g
$NaH_2PO_4.H_2O$	4 g

Na_2HPO_4 and $NaH_2PO_4.H_2O$ were dissolved in 900 ml of deionized water and formaldehyde added to 1L.

9.2 Appendix 2

9.2.1 Publications arising from this research

Hill, P.A., Tumber, A., Meikle, M.C. (1997) Multiple extracellular signals promote osteoblast survival and apoptosis. Endocrinology. **138(9)**:3849-3858.

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